Comprehensive analysis of pulmonary Large Cell Neuroendocrine Carcinoma (LCNEC)

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new insights to guide diagnosis and treatment

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Comprehensive analysis of pulmonary Large cell neuroendocrine carcinoma (LCNEC)

new insights to guide diagnosis and treatment

PROEFSCHRIFT

Ter verkrijging van de graad doctor aan de Universiteit Maastricht,
Op gezag van de Rector Magnificus, prof. dr. mr. Rianne M. Letschert
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 8 december 2017, om 14.00 uur

door

Jules Louis Derks

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Dr. M.M.H. Hochstenbag
Prof. dr. W. Timens (University of Groningen and University Medical Centre)

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Chapter 1

General introduction

General introduction and outline of this thesis

Pulmonary neuroendocrine tumors represent a subgroup of lung cancer likely arising from neuroendocrine cells of the bronchopulmonary epithelium. Approximately 20% of all lung cancers are of neuroendocrine origin. While these tumors share similar morphologic and immunohistochemical features, they are characterized by differing biologic behavior, ranging from low to high-grade aggressive tumors. Typical carcinoids are low-grade, slow growing malignancies that rarely metastasize, atypical carcinoids are intermediate-grade malignancies, and large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancers (SCLC) are high-grade carcinomas with at diagnosis frequently metastatic disease[1]. Cancer registries have reported an increase in incidence of carcinoid (1-2%) and LCNEC (1-3%) whereas incidence of SCLC is decreasing (≈20%)[1-3].

Large cell neuroendocrine carcinoma (LCNEC) is an uncommon tumor of the lung, with a reported incidence of approximately 3% in surgical case series[4]. Due to a high degree of diagnostic difficulty and a low incidence, many aspects of this tumor type are still unknown.

1. Pathological diagnosis of LCNEC

Diagnostic classification and nomenclature of neuroendocrine lung tumors has changed regularly since the introduction of carcinoid disease in 1907. Before the introduction of LCNEC, neuroendocrine tumors of the lung were subdivided in three classes; the typical carcinoids, atypical carcinoids and SCLC, the complete diagnostic history for lung tumors is provided in Figure 1.1

After reviewing a set of neuroendocrine lung tumors (carcinoids and SCLC), Travis and colleagues found in 1991 a group of tumors that deviated from the known classification in morphology from SCLC but had similar prognosis[5]. This new group was classified as a high-grade neuroendocrine non-small cell carcinoma and placed in-between atypical carcinoid and SCLC. Since 1999, the diagnostic classification criteria for neuroendocrine tumors have remained unchanged. However in the recent WHO classification LCNEC is moved from the NSCLC category to that of neuroendocrine lung tumors[1]. Figure 1.2 outlines the most recent WHO/IASLC classification established in 2015[1].

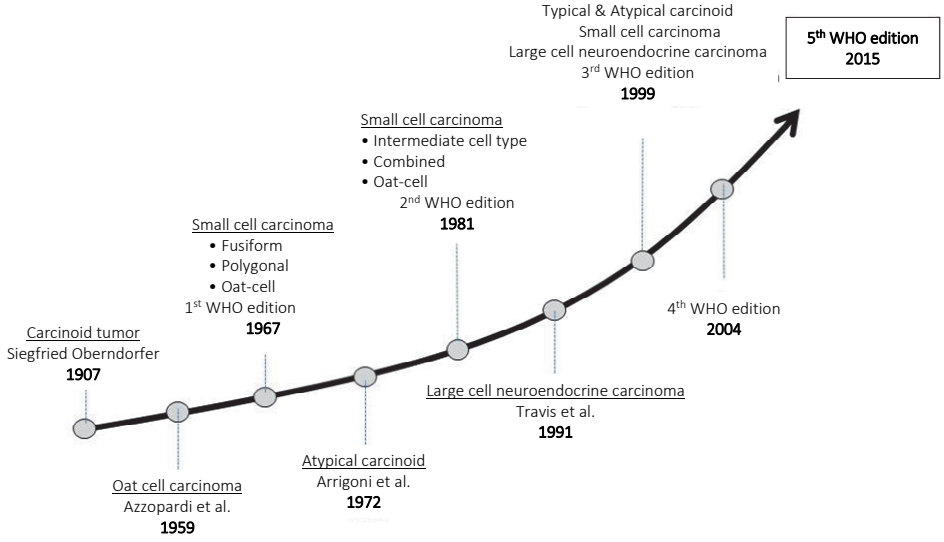

Figure 1.1 The diagnostic nomenclature for neuroendocrine tumors from 1907 to the 5th World Health Organization classification (2015) is presented. From 1999 to 2015 LCNEC was classified as a non-small cell lung carcinoma (NSCLC); however, since 2015 LCNEC is categorized as a neuroendocrine tumor

Diagnostic classification criteria for LCNEC include a high proliferative rate with >10 mitosis per 2mm² on high power field (HPF) and areas of abundant necrosis, neuroendocrine morphology and immunohistochemical expression of neuroendocrine markers (chromogranin-A, synaptophysin or NCAM/CD56). The cytological features of LCNEC resemble those of non-small cell lung cancer (NSCLC) with large cell size and abundant cytoplasm and (occasional) nucleoli.

The diagnosis of LCNEC is complex and often requires a large surgical resected lung biopsy to enable the evaluation of mitosis and neuroendocrine morphology. Furthermore, a moderate inter-observer variation with kappa ranging from 0.35 to 0.81 has been reported for LCNEC *versus* SCLC[6-8]. The most commonly overlapping diagnoses with LCNEC are carcinoid and SCLC while only in some cases NSCLC is reported. Data on the accuracy and precision of a diagnosis of LCNEC established on biopsy samples are not available[9-11]. In Figure 1.2 the diagnostic overlap and criteria used to separate LCNEC from carcinoid (mitotic rate >10), from NSCLC (observation of neuroendocrine morphology) and from SCLC (large cell size, cytoplasm) are presented.

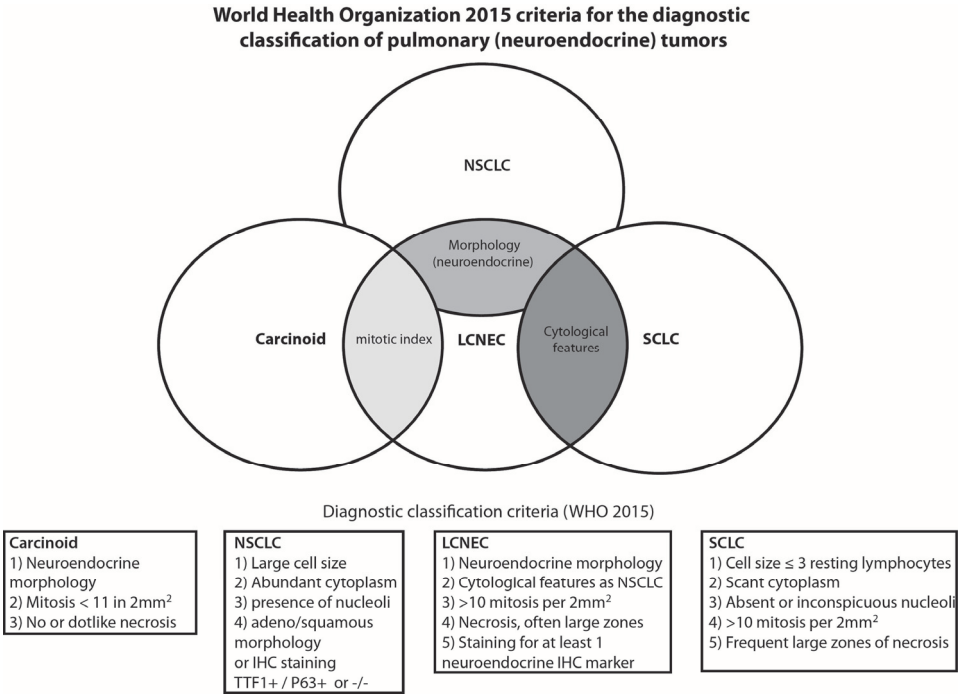

Figure 1.2 The World Health Organization diagnostic classification (2015) for pulmonary (neuroendocrine) tumors is presented
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; WHO, World Health Organization; mm, millimeter; IHC, immunohistochemical

2. Clinical characteristics and treatment of LCNEC

The majority of patients diagnosed with LCNEC are middle aged to elderly males with a history of heavy smoking[4]. Symptoms observed in patients with LCNEC are comparable to other lung cancers including hemoptysis, coughing and rapid weight loss[12]. On radiological evaluation LCNEC is predominantly found in the lung periphery[13]. The uptake of fluorine 18-fluorodeoxyglucose (FDG) evaluated by positron emission tomography (PET) scan is high and an effective tool to diagnose metastatic spread of LCNEC[14,15].

Despite the requirements of LCNEC to be diagnosed on surgical resected tissue usually available in stage I-II disease, LCNEC is diagnosed in stage III or IV disease in over 66%[16]. Curation is only possible in early stage disease by radical surgery or radiotherapy. After surgery, the five-year survival of patients is low, ranging from 28-57%[17]. Studies

investigating the beneficial effects of adjuvant chemotherapy in LCNEC lack statistical power and design to establish any firm conclusions[4,18-21].

The optimal systematic treatment for LCNEC is debated. Current guidelines favor SCLC based chemotherapy regimens, such as platinum-etoposide, being grounded on expert opinion[22]. Nevertheless, LCNEC is a tumor with NSCLC features and the response rate to platinum-etoposide chemotherapy is reportedly lower than observed in similarly treated SCLC disease[23,24]. Therefore, several clinicians favor NSCLC type chemotherapy regimens including gemcitabine, taxane compounds and pemetrexed for LCNEC. In LCNEC, SCLC type chemotherapy regimens have been evaluated in two phase II trials and reported a median survival ranging from 8.0 (3.7-7.9) to 12.6 (9.3-16.0) months, and an objective response rate of 34-47%[23,24]. Several retrospective studies have evaluated chemotherapy in LCNEC and reported varying results favoring either NSCLC or SCLC type regimens[19,25,26].

In conclusion LCNEC is a highly aggressive disease frequently diagnosed at time of stage IV disease. Optimal systematic treatment for LCNEC is unclear, platinum-etoposide seems to be the most effective treatment but comparative studies with NSCLC type chemotherapy treatment are lacking.

3. The molecular background of LCNEC

Genomic studies have started to unravel the molecular defects causing LCNEC, and provided evidence that gene mutations and gene expression patterns found in LCNEC are also frequently found in SCLC but are different from carcinoids[27]. Gene expression profiling array studies in LCNEC and SCLC identified unique gene clusters; these clusters were not correlated with histological classification into LCNEC or SCLC[28,29] suggesting that the current classification does not follow biological tumor behavior. Genes identified to have different expression level includes high in LCNEC for *Vil1* and *CDX-2*, frequently expressed in colorectal adenocarcinomas, and high in SCLC but low in LCNEC for *BAI3*[30].

Several studies have investigated the mutational background of LCNEC in a targeted approach and, different than from NSCLC, Kirsten rat sarcoma viral oncogene homolog (*KRAS*), epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (*ALK*) rearrangement were rarely identified[31,32]. Whole-exome sequencing of 15 LCNEC tumors showed overlapping mutations between LCNEC and SCLC including the

genes *TP53, RB1*, and *EP300*[33]. The *TP53* gene, which helps to maintain genomic stability, is mutated in approximately 80% of LCNECs[34,35]. Moreover, the *p16/cyclin D1/ Rb1* pathway is affected in 62%[36].

In conclusion, the available data suggest that LCNEC is a high-grade neuroendocrine carcinoma that is closely related to SCLC disease making it conceivable that LCNEC should be treated in a similar fashion as SCLC patients.

4. Aims and outline of this thesis

In this thesis, we examined the epidemiological, clinical, histopathological, and molecular features of LCNEC. We studied establishment of diagnosis in the Netherlands, especially on biopsy specimens, from 2003-2012. Also, we evaluated how we could improve the diagnosis LCNEC established on biopsy samples. Finally, we evaluated changes in chemotherapy treatment and outcome for types of chemotherapy in LCNEC separately and in combination with recently identified genomic LCNEC subtypes. Combined these analyses should enable a randomized controlled trial evaluating optimal systematic treatment in patients with LCNEC disease. An overview of the aims is provided in Figure 1.3.

Chapters 2-8 of this thesis evaluated the clinical characteristics and diagnosis of LCNEC in the Netherlands. In Chapter 2 diagnosis, clinical characteristics, and treatment of LCNEC is extensively reviewed. In Chapter 3 the incidence, clinical characteristics, and treatment of LCNEC are compared to SCLC and NSCLC, subdivided into squamous cell carcinomas (SqCC) and adenocarcinoma (AdC) in the Netherlands from 01-01-2003 to 31-12-2012. Chapter 4 describes a case report highlighting the difficulty and importance of correct classification of neuroendocrine lung tumors. In Chapter 5 we subsequently evaluate the application of nomenclature for neuroendocrine lung tumors, including LCNEC, in the daily routine pathology practice by evaluation of the Pathology Registry (PALGA). In Chapter 6 we examine the description of the diagnostic WHO 2004/2015 criteria for LCNEC in pathology reports with a conclusive LCNEC diagnosis compared to WHO criteria identified by panel review. In Chapter 7 we address a problem occurring in the daily pathology practice regarding NSCLC with neuroendocrine immunohistochemical differentiation not addressed by the WHO 2015 classification. In Chapter 8 we subsequently compared paired pre-operative biopsies of surgically confirmed LCNEC to investigate the accuracy and precision of LCNEC diagnosed on a biopsy specimen.

Chapter 9 focusses on improvement of treatment of LCNEC in the Netherlands. Temporal changes in chemotherapy treatment in the Netherlands for LCNEC, and (optimal) response to subtypes of chemotherapy regimens in pathology revised LCNEC is discussed. Also, we evaluated clinical relevance of newly established genomic markers for recently identified genomic signatures of LCNEC in Chapter 10. Finally, in Chapter 11, we discuss the rapidly evolving field of genomics in neuroendocrine lung tumors, in specific that of the large cell neuroendocrine carcinoma, and evaluate expected future treatment options.

We further elaborate on new insights for the diagnosis and treatment of LCNEC in the general discussion (Chapter 12) combined with the potential implications of recent genomic findings. Finally, a summary of the dissertation is provided.

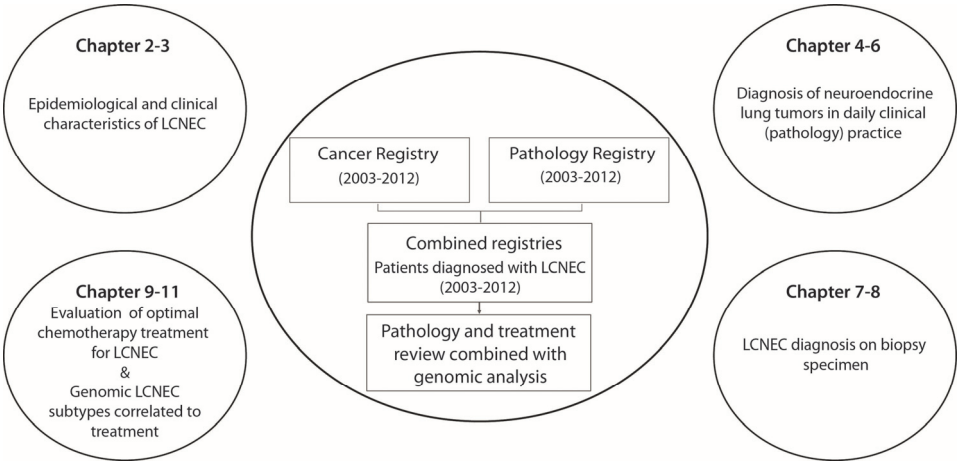

Figure 1.3 Outline of the thesis "Comprehensive analysis of pulmonary Large Cell Neuroendocrine Carcinoma *New Insights to guide Diagnosis and Treatment*"
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma

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Chapter 2

Adapted from: The IASLC multidisciplinary approach to thoracic oncology

Chapter 55: Neuroendocrine tumours of the lung other than small cell lung cancer

K. Noonan, J.L. Derks, K. Laskin, A-M.C. Dingemans

Pulmonary neuroendocrine tumors represent a spectrum of tumors that develop from neuroendocrine cells of the bronchopulmonary epithelium. Although neuroendocrine tumors have similar morphologic and immunohistochemical (IHC) features, they span a broad clinical-pathologic spectrum and are characterized by differing biologic behavior. Typical carcinoids are low-grade, slow-growing malignancies that rarely metastasize; atypical carcinoids are intermediate-grade malignancies; and large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancers (SCLC) are high-grade carcinomas[1].

LCNEC is an uncommon tumor of the lung with an incidence of approximately 3%, as reported in surgical case series[2]. LCNEC has only recently been described as a form of high-grade lung cancer that expresses neuroendocrine features. Because of the low incidence, evolving classification, and high degree of diagnostic difficulty, many aspects of LCNEC are still unknown.

Classification

Before the introduction of LCNEC, neuroendocrine tumors of the lung were subdivided into three classes: typical carcinoids, atypical carcinoids, and SCLC. In 1991, after reviewing a set of neuroendocrine tumors (typical and atypical carcinoids and SCLC), Travis et al. found a group of tumors that deviated from the known classification in prognosis and morphology. They classified this new group as a high-grade neuroendocrine non-small cell carcinoma (NSCLC) and placed it in between atypical carcinoid and SCLC[3].

In 1999, LCNEC was recognized in the World Health Organization (WHO) classification of lung tumors as a large cell carcinoma based on its cytological parallels (large cell size and abundant cytoplasm). LCNEC is different from other large cell carcinomas because of its combination of neuroendocrine differentiation and morphology. Other large cell carcinoma histology can express neuroendocrine morphology, but not in combination with neuroendocrine differentiation. Other regularly used terms referring to LCNEC are neuroendocrine carcinoma (NEC) grade 3, poorly differentiated NEC, and high-grade NSCLC; however, these terms are also used for SCLC and thus may include tumors with SCLC and/or LCNEC histology.

The most recent WHO/International Association for the Study of Lung Cancer (IASLC) classification of neuroendocrine lung tumors was established in 2004 by Travis et al. (Table 2.1)[1].

Table 2.1 Diagnostic criteria of pulmonary neuroendocrine tumors ***(WHO 2004 guidelines*)***

Typical carcinoid
A tumor with carcinoid morphology and less than 2 mitoses per 2mm² (10 HPF), lacking necrosis and 0.5 cm or larger
Atypical carcinoid
A tumor with carcinoid morphology with 2-10 mitosis per 2mm² (10 HPF) OR necrosis (often punctate)
Large cell neuroendocrine carcinoma
A tumor with a neuroendocrine morphology (organoid nesting, palisading, rosettes, trabeculae)
High mitotic rate: 11 or greater per 2mm² (10 HPF), median of 70 per 2mm² (10 HPF)
Necrosis (often large zones)
Cytologic features of a non-small cell carcinoma: large cell size, low nuclear to cytoplasmic ratio, vesicular, coarse, or fine chromatin, and/or frequent nucleoli. Some tumors have fine nuclear chromatin and lack nucleoli, but qualify as NSCLC because of large cell size and abundant cytoplasm.
Positive immunohistochemical staining for one or more NE markers (other than neuron specific enolase) and/or NE granules by electron microscopy.
Small cell carcinoma
Small size (generally less than the diameter of 3 small resting lymphocytes)
Scant cytoplasm
Nuclei: finely granular nuclear chromatin, absent or faint nucleoli
High mitotic rate (11 or greater per 2mm² (10 HPF), median of 80 per 2mm² (10 HPF)
Frequent necrosis often in large zones

*Originally printed in Travis et al. World Health Organization Classification of Tumors; Tumors of the Lung, Pleura, Thymus and Heart, IARC press 2004[1]

Abbreviations: HPF, high power field; NSCLC, non-small cell lung cancer; NE, neuroendocrine

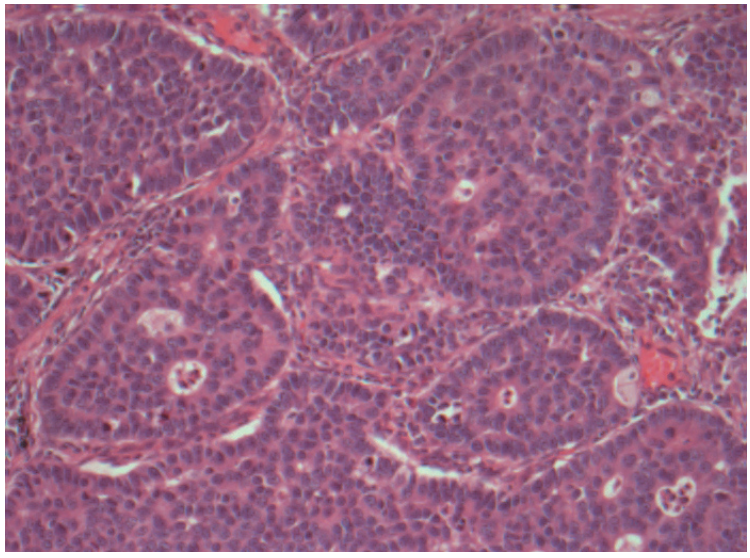
Diagnosis

Histology

LCNEC

The diagnosis of LCNEC is complex and often requires a large surgically resected lung biopsy, mainly because small biopsies are susceptible to crushing artefacts that may disturb the neuroendocrine morphology and cell size, two features that are critical for the diagnosis of LCNEC. To establish an LCNEC diagnosis, several histologic criteria should be confirmed (Table 2.1). LCNEC express a neuroendocrine growth pattern (morphology) that is similar to that seen in low-grade neuroendocrine tumors (carcinoids). On slides stained with hematoxylin and eosin, these neuroendocrine growth patterns are recognized as organoid nesting, trabeculae, rosette-like structures, or palisading cells (Figure 2.1A & 2.1B).

A



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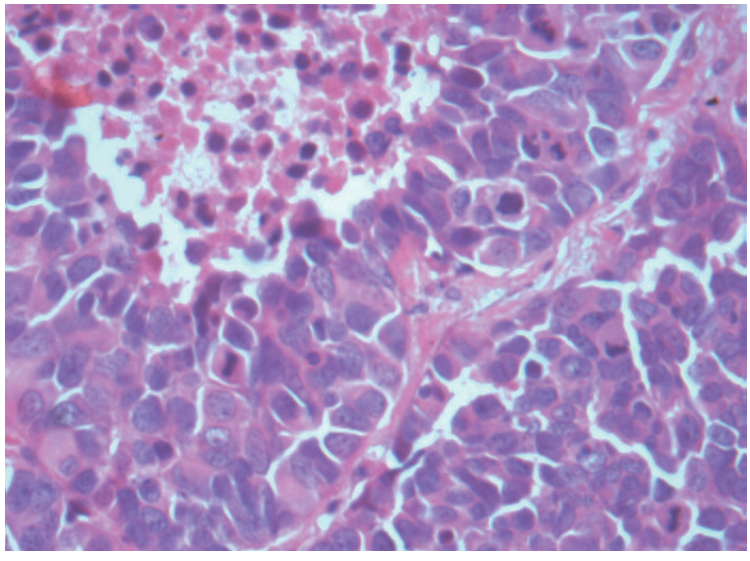


Figure 2.1 **A**) Large cell neuroendocrine carcinoma (H&E, x100). Nests of tumor cells with peripheral palisading and central necrotic foci and rosette-like structures. **B**) High magnification view (H&E, x400). Note large cells with abundant eosinophilic cytoplasm and round to oval nuclei with a fine granular ("salt-and-pepper") to more clumped chromatin with occasional nucleoli. Numerous mitotic figures and necrosis (upper left corner) are present. (Courtesy of Dr. M. Béndek, Maastricht University Medical Centre.)

All LCNECs have a high proliferative rate, with more than 10 mitoses/mm² on HPF examination. Total HPF examination should include an area of 2 mm², preferably in the regions with the highest mitotic activity and viable cells. Besides high mitotic activity, areas of necrosis are frequently noted. By definition, all LCNECs express neuroendocrine differentiation, which is immunohistochemically confirmed when focal activity (more than 10% positive cells) is found with use of a neuroendocrine markers (chromogranin, synaptophysin, or neural cell adhesion molecule [CD56]) (Figure 2.2A & 2.2B). Presence of neuroendocrine granules on electron microscopy examination is also sufficient for diagnosis of LCNEC. The morphologic features of LCNEC resemble those of NSCLC, because the cells are large with abundant eosinophilic cytoplasm and a low nuclear-to-cytoplasmic ratio. The nuclei are round to oval-shaped with granular chromatin (so-called salt and pepper). Nucleoli are also frequently seen (Figure 2.1A & 2.1B).

Cytology

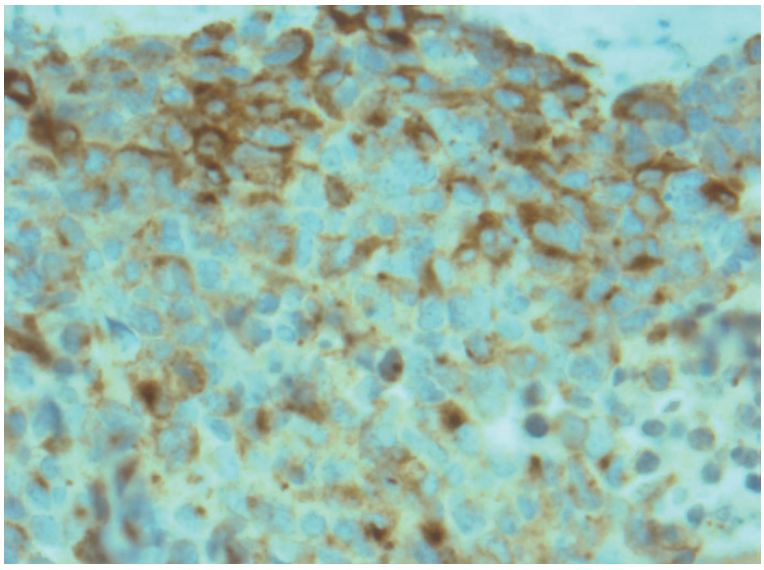
Although cytological smears are not suitable for establishing a diagnosis of LCNEC, cytological examination can be useful during the initial evaluation of a tumor. On cytological smears, the presentation of LCNEC is medium- to-large round or polygonal cells arranged in groups or as a single cell. The LCNEC cells can be arranged in rosette-like structures or peripheral palisading cells, and nuclear molding can be seen. In the background of the cytological smears, necrosis and nuclear streaking is commonly seen. LCNEC cells have scant or moderate amounts of cytoplasm with a high nuclear-to-cytoplasmic ratio, which is dependent on the fixation material (air-dried vs. alcohol fixated)[4]. Most often, the nuclear shape is round or oval and nuclear mitosis are frequently found. Nuclear pleomorphism and nucleoli are occasionally present.

Differential diagnosis

Histology

Diagnosing LCNEC is a highly complex process and was addressed in inter-observer studies on resected pulmonary neuroendocrine tumors. In a study performed by a panel of expert lung pathologists, a moderate inter-observer variation with kappa ranging from 0.35 to 0.81 was reported[5-7]. The most common overlapping diagnoses with LCNEC were SCLC, LCC, atypical carcinoid, basaloid carcinoma, and the poorly differentiated NSCLC tumors expressing a neuroendocrine phenotype[2,6]. Undoubtedly, these uncertainties are secondary to the morphologic and cytological similarities of lung tumors with LCNEC.

A



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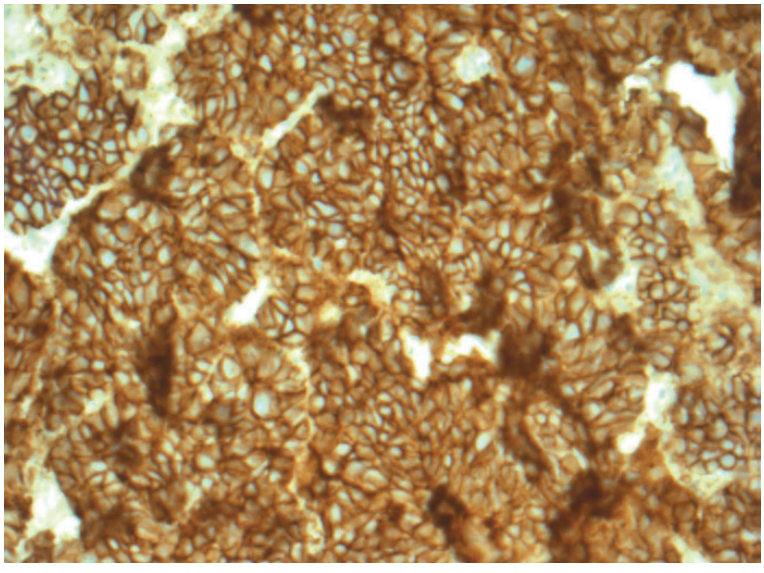

Figure 2.2 Immunohistochemistry of a pulmonary large cell neuroendocrine carcinoma. **A**) Granular cytoplasmic staining with Chromogranin-A (x100) and **B**) diffuse membrane positivity of CD56 (NCAM, x100) can be observed. (Courtesy of Dr. M. Béndek, Maastricht University Medical Centre.)

Although differentiating pulmonary neuroendocrine carcinomas (NEC) from overlapping tumors can be a challenge, several criteria may help guide the diagnosis. The differentiation of atypical carcinoid from high-grade NEC can be achieved with the help of the mitosis count; on average, 2 to 10 mitoses/2 mm^2 are expressed in atypical carcinoid compared with more than 11 mitoses/2 mm^2 in high-grade NEC. Moreover, necrosis in atypical carcinoid predominantly consists of punctate foci, whereas necrosis is more prominent in LCNEC[8].

A distinction between LCNEC and SCLC can be established only with the help of cytology-based criteria. Compared with LCNEC, SCLC has a smaller cell size (less than the diameter of three lymphocytes), a higher nuclear: cytoplasmic ratio, and absent or faint nucleoli. Although it was demonstrated in several studies that LCNEC and SCLC had a considerably different mean cell size, the standard deviations did overlap. Therefore, whether cell size is the most adequate criteria to differentiate LCNEC from SCLC should be questioned[9-11]. The mitosis count, which is useful in atypical carcinoid, is not helpful in differentiating LCNEC from SCLC.

In addition to atypical carcinoid and SCLC, other forms of non-small carcinomas—such as the basaloid carcinoma—must be distinguished from LCNEC. The morphology of basaloid carcinoma is comparable to that of LCNEC; differentiation is possible with the help of IHC with neuroendocrine markers, which are negative in basaloid carcinomas[1]. As for the basaloid carcinoma, poorly differentiated adenocarcinomas or squamous cell carcinomas can also be distinguished from LCNEC with the help of IHC markers, which are addressed later in the chapter.

Cytology

Compared with histology, misdiagnoses are even more common when LCNEC is diagnosed on cytology specimens. Preoperatively obtained cytological smears from confirmed resected LCNEC have been reviewed in several studies[4,12,13]. In about 80% of smears, the cytology was initially diagnosed as NSCLC, SCLC, or large cell carcinoma with neuroendocrine features, poorly differentiated adenocarcinoma or squamous cell carcinoma, or combined SCLC. However, during the period between 1990 and the beginning of 2000, when most of the series in these studies were diagnosed, LCNEC was a new entity and pathologists may have been unaware of it. In 2005, a study was published in which nine (90%) of 11 LCNEC cases (N=11) were correctly diagnosed before surgery[13].

Cytokeratin markers

High-molecular-weight cytokeratin (CK) types 1, 5, 10, and 14 (antibody clone 34E1β2) are almost solely expressed in non-neuroendocrine carcinomas such as basaloid carcinoma and poorly differentiated adenocarcinoma or squamous cell carcinoma. In tissue microarray panel studies, 3% to 17% of pure LCNEC showed positive staining for clone 34E1β2[10,14,15]. Other commonly used high-molecular-weight CK types such as CK5/6, CK7, and CK20 were positive in 2% to 13%, 57% to 77%, and 2% to 10% of LCNECs, respectively[16,17]. CK18 and CK19 were positive in 97% and 59%, respectively[16,17]. In combined LCNEC, expression of 34E1β2 has been noted in the adenocarcinoma/squamous cell carcinoma component[14].

Markers for differentiating LCNEC from squamous cell carcinoma

In addition to high-molecular-weight CKs, markers such as desmocollin-3 and p63 may be useful in discriminating poorly differentiated squamous cell carcinomas from LCNEC. Desmocollin-3 was negative in 99% of all pure LCNECs and therefore appears to be a promising specific marker but requires further validation[16]. Expression of p63, a highly sensitive and specific marker for diagnosing squamous cell carcinomas, was detected in 0% to 18% of LCNECs. Therefore, p63 may not be appropriate as a specific marker for poorly differentiated NSCLC when LCNEC is a possibility in the differential diagnosis[10,16,18]. When compared with p63, the non-trans activating isoform of the *p63* gene, delta Np63 (p40), was found to be a more reliable marker for squamous differentiation. Expression of p40 was lower compared with p63 in LCNEC and therefore more accurate[19]. These results were confirmed in a study subtyping LCC with IHC; none of the confirmed LCNECs stained for p40[20].

Markers for differentiating LCNEC from adenocarcinoma

Thyroid transcription factor-1 (TTF-1), a marker commonly used in defining the appropriate histotype of lung cancer and that is also sensitive and specific for lung adenocarcinoma, is expressed in about 50% of all resected lung LCNEC. The expression ranges of TTF-1 mainly depend on the antibody used. The more sensitive clone, SPT24, is positive in 23% to 77% of LCNECs[14,17,21], and the 8G763 clone is positive in 23% to 48%[14,16,21,22]. Therefore, TTF-1 is not useful in differentiating LCNEC from poorly differentiated NSCLC (adenocarcinoma). A new marker named Napsin-A, specific to adenocarcinomas, was negative in 100% of LCNECs, but further validation of this marker is required[16]. In addition, collapsin response mediator protein (CRMP) is known to be involved in neurogenesis and was studied in a group of lung tumors. CRMP was expressed in four (100%) of four LCNECs and 54 (100%) of 54 SCLCs. The expression of

CRMP was negative in 0% of 22 adenocarcinomas and positive in one (8%) of 12 squamous cell carcinomas. The number of LCNECs studied was very small, but CRMP was shown to be a promising marker that also requires further validation[23].

Markers for differentiating LCNEC from SCLC

As of the time of publication, distinguishing LCNEC from SCLC by IHC is not feasible. Several markers have been proposed, but lack in practical usefulness. Both CK7 and 18 are commonly expressed in LCNEC and were reported in several studies to have a considerably higher expression intensity in LCNEC compared with SCLC[17,24] These results were similar in studies comparing the expression intensity of E-cadherin and β-catenin in LCNEC and SCLC, where both markers were shown to have considerably higher expression intensity in LCNEC[17,24]. Villin1, a promising marker located in the brush border of epithelial cells, was found to be expressed in 62% of LCNECs and 4% of SCLCs[24,25]. However, these results must be confirmed in larger studies. In a smaller study, IHC expression of neuronatin was examined in LCNEC and SCLC after an increased cDNA transcription of neuronatin in LCNEC was noted. Among the SCLC samples, 8% were positive for neuronatin with IHC staining compared with 43% among the LCNEC samples[26]. A neurogenesis-regulating gene (*NeuroD*) was also found to be differentially expressed between LCNEC and SCLC[10]. *NeuroD* was expressed in 53% of LCNECs and 13% of SCLCs. Therefore, several markers may differentiate LCNEC from SCLC, but none of these is very specific or sensitive and further validation is required.

Markers for differentiating atypical and typical carcinoids from LCNEC

Separating atypical and typical carcinoids from LCNEC is possible with the Ki-67 index. The expression index in LCNEC is approximately 40% (range, 25% to 52%); for carcinoids, the expression index is reported to be below 20% (typical carcinoid, less than 2%; atypical carcinoid, less than 20%, typically ± 10%)[27-30]. Consequently, Ki-67 may be useful in differentiating LCNEC from atypical and typical carcinoid, although the diagnosis should be confirmed with a mitoses count.

IHC on cytology

IHC neuroendocrine markers such as chromogranin, synaptophysin, and NCAM were found to be positive in 28% to 31%, 64% to 75%, and 45% of cytological smears, respectively[4,31].

Molecular biology

The molecular basis of LCNEC is also still unknown. SCLC and LCNEC are known as highly undifferentiated pulmonary tumors that express similar characteristics. However, according to WHO classification, LCNEC is categorized as a subtype of LCC. The molecular biology of LCNEC, determined by different techniques, has been addressed in several studies and the findings compared with SCLC and LCC.

Loss of heterozygosity (LOH)

In an evaluation of LOH with microsatellite markers at chromosome 3p, 5q, 9p, 11q, and 13q, several similarities between SCLC and LCNEC were demonstrated[32]. These chromosomal findings were in accordance with the results from another study that compared LOH in large cell carcinoma, LCNEC, and SCLC[33]. LOH was examined for chromosome 3p, 5q, 9p, 10p, 10q, and 13q. Significant differences were noted between all three tumor types except for SCLC and LCNEC, emphasizing the close relation between these high-grade neuroendocrine tumors. LOH has also been examined in combined SCLC and LCNEC tumors. Depending on the region being examined, combined tumors can express an SCLC or LCNEC phenotype. Investigators separately studied SCLC and LCNEC regions, and found a high degree of similarity in the genetic profile. These findings suggest that there is a common origin of combined SCLC and LCNEC tumors[34].

Chromosomal aberrations

CGH and other genetic analyses to assess chromosomal aberrations in BP-carcinoids[35-40], high-grade pulmonary neuroendocrine carcinomas,[41-45] or both[45-48] have been examined in several studies. Swarts et al. performed a meta-analysis of these studies, which included 87 typical carcinoids, 38 atypical carcinoids, 33 LCNECs, 48 SCLCs, and 11 unclassified high-grade neuroendocrine tumors. Chromosomal aberrations more than 10 Mb were much more frequent in LCNEC (average 13.7 aberrations per tumor) and SCLC (18.8 aberrations), as compared with atypical carcinoids (6.1 aberrations) and typical carcinoids (2.8 aberrations). The investigators assumed that smoking habit may explain the differences. The most frequent aberrations for pulmonary carcinoids are -11q, +19P, -13q, +19q, +17q, -11p, -6q, +16p, +20p, and -3p. These aberrations differ from those present in the high-grade neuroendocrine tumors, except for 3p and 13q losses[49].

Through CGH, loss of chromosome 3p, 4, 5p, 6q, 8p, 9p and 21q, and gain in 5p, 8q, 12p and 22, was found in three patients with LCNEC. Similarities between chromosomal aberrations in LCNEC and SCLC included loss of 3p, 4q, 5q, and 13q and gain of 5p.

Noticeable chromosomal aberrations between SCLC and LCNEC were found at chromosome arm 3q (gain), 10q (loss), and 17p (loss) in favor of SCLC, and gain of 6p in favor of LCNEC. Compared with atypical carcinoid, no similarities were detected. In a study by Peng et al., several similarities between LCNEC and SCLC were found when the genetic profiles were analyzed with high-density bacterial artificial chromosome array Frequently gained loci were located at 1q, 2q, 3q, 5p, 7q, 8q, 12q, and 18q; lost loci were located at 1p, 3p, 4q, 5q, 10q, 13q, 16q, 17p, and 22q. Considerably different chromosomal aberrations compared between all stages of SCLC and LCNEC were located at 2q (gain), 3p (loss), 4q (loss), and 6p (loss). Although it may appear that SCLC and LCNEC share a common genetic profile, most similarities may not be very specific, because numerous chromosomal aberrations are commonly encountered in other forms of lung cancer, including pulmonary neuroendocrine carcinoma.

Microarray comparison

Anbazhagan et al. performed hierarchical clustering of gene expression profiles of two carcinoids, two SCLCs, and two brain tumors. Although the carcinoids clustered together with the brain tumors, SCLC was more closely related to normal bronchial epithelial carcinoma[50]. When examining the clinical behavior of high-grade neuroendocrine tumors, classification based on genetic profiling may be more appropriate than histologic classification. Jones et al. examined cDNA microarray data obtained from neuro-endocrine lung tumors (eight LCNECs [two combined with SCLC], 17 SCLCs [two combined with LCNEC], and 13 large cell carcinomas). Surprisingly, neither SCLC nor LCNEC clustered as single entities in the way that large cell carcinomas did[51]. In support of this molecular description of neuroendocrine lung tumors, Shibata et al. found three high-grade neuroendocrine subclasses when data from comparative genome hybridization were hierarchically clustered (eight SCLC, 15 LCNEC). The three subclasses branches were subdivided in groups named BR1, BR2, and BR3. Patients in the BR2 group had a significantly different survival compared with the BR1 and BR3 groups (P=0.028). However, contrary to findings from Jones et al., almost all SCLCs clustered[52].

Mutation analysis

MEN1 is an autosomal dominant disorder associated with mutations in the gene locus on 11q13. *MEN1* gene activation is evident in 70% of patients with atypical carcinoid, 47% with typical carcinoid, 52% with LCNEC, and 41% with SCLC. A single mutation was found in a small study of MEN1 in LCNEC[53].

Capodanno et al. assessed 190 patients with pulmonary neuroendocrine tumors (75 typical carcinoids, 23 atypical carcinoids, 17 LCNECs, and 75 SCLCs) and found that there was an increasing frequency of *PI3K* mutations, with increased biologic aggressiveness of the pulmonary neuroendocrine tumors, except in LCNECs[54]. *PIK3CA* mutations were present in 13% of typical carcinoids, 39% of atypical carcinoids, 31% of SCLCs, and in only 12% of LCNECs.

Mutations in LCNEC that are commonly encountered in lung cancer have been examined in several studies (Table 2.2). Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and epidermal growth factor receptor (*EGFR*) mutations are uncommon in LCNEC, although there have been published reports in cases of combined LCNEC and adenocarcinomas[32,55,56]. Aberrant expression of anaplastic lymphoma kinase (ALK) by IHC was demonstrated in a single case of LCNEC. No *ALK* rearrangement or mutation was noted in further analyses[57]. Two *PIK3CA* mutations have been found at c.3145 G>A and c. 3140 A>G.95 In 29% of resected LCNECs, a mutation in the neurotropic tyrosine receptor kinase gene family was demonstrated[55].
In a 2013 study, a large group of molecularly analyzed lung tumors was reported by the Clinical Lung Cancer Genome Project and Network Genomic Medicine[58]. A total of 261 lung tumors (31 large cell carcinomas) were included for unsupervised hierarchical clustering of gene-expressions. Although the analyzed carcinoid tumors harbored no noteworthy mutations, several mutations were identified in pure LCNEC and the combination of SCLC and LCNEC (Table 2.2). Based on genetic modelling, the authors of the study could classify most all large cell carcinomas as adenocarcinoma, squamous cell carcinoma, or SCLC. Thereby, the diagnosis of large cell carcinoma as a separate entity was brought into question. Whole-exome (15 tumors) and transcriptome (10 tumors) sequencing of LCNEC showed overlapping mutations between LCNEC and SCLC (*T53*, *RB1*, and *EP300*). Several mutations typically found in adenocarcinomas or squamous cell carcinomas were detected in LCNEC, but these findings were not significant. Additional information about the whole-exome and transcriptome sequencing of LCNEC is expected to be published in the near future[58].

Table 2.2 Overview of all mutations analyzed in pure and combined LCNEC (only published data)

	LCNEC/SCLC	*LCNEC*	*LCNEC/SqCC*	*LCNEC/AdC*
ALK	*	0/106 [i]	*	*
BRAF	2/9	0/24	0/3	*
EGFR	0/10	1/62	0/3	0/8
KRAS	1/10	2/83	*	1/9
NRAS	0/6	0/34	*	*
PIK3CA	1/10	2/43	*	*
ROS1	*	*	*	*
HRAS	0/8	1/17	0/3	*
MEN1	*	1/13	*	*
TP53	3/10	32/61	*	1/1
KEAP1	0/9	2/19	0/1	*
NTRK	*	6/21	*	*
NFE2L2	0/9	1/19	*	*
STK11	1/10	8/28	*	*
CDK4	0/10	0/28	0/3	*
DDR2	0/2	0/1	0/1	*
ERBB2	0/10	0/26	0/3	*
FGFR2	0/10	1/24	0/1	*
FGFR3	0/9	0/25	0/2	*
C-Kit	*	0/83	*	*
C-met	*	0/83	*	*
PDGFRα	*	0/83	*	*
PDGFRβ	*	0/83	*	*

* No data has been reported about this mutation. [i] Immunohistochemical analyses of ALK expression
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell carcinoma; SQ, squamous cell carcinoma; AD, adenocarcinoma

Pathways

The *p53* gene, which helps to maintain genomic stability, is mutated in approximately 4% of typical carcinoids, 29% of atypical carcinoids, 80% of LCNECs, and 75% of SCLCs[32,59-64].

The *p16/cyclin D1/ RB1* pathway is affected in 9% to 20% of typical carcinoids, 22% of atypical carcinoids, 62% of LCNECs, and 71% to 90% of SCLCs[30,40,64-69]. The *RB1* gene is a tumor suppressor with a critical role in cell cycle control through the regulation of the G1 growth arrest. The *P16* gene, is a cyclin-dependent kinase inhibitor that inhibits binding of cyclin-dependent kinases to Cyclin D1 and prevents phosphorylation (inhibition) of RB1. Therefore, down regulation of *P16* and *RB1* may lead to uncontrolled cell growth. Loss of RB1 expression was demonstrated in 47% to 91% of LCNECs. Loss of P16 expression and overexpression of Cyclin D1 were found more frequently in LCNEC than in SCLC[10,29,30,33,70].

The intrinsic apoptosis pathway, including *Bcl2*, *Bcl2L1*, and *BAX* genes, has been found in several studies to be inhibited in high-grade neuroendocrine tumors, moderately affected in atypical carcinoids, and almost intact in typical carcinoids[71,72].

The expression of AKT and mammalian target of rapamycin (mTOR) as part of the *PI3K/AKT/mTOR* pathway in a group of neuroendocrine lung tumors has also been evaluated in several studies; however, there were contradicting results for AKT expression (19% to 82%) and mTOR expression (50%-77%) in these tumors[73,74]. Righi et al. described a pattern of mTOR intensity expression that decreased from low- to high-grade neuroendocrine tumors. The authors also noted that mTOR expression correlated with SSRT-2/3 expression, and hypothesized that mTOR may be a possible regulator of SSRT expression[75].

Clinical characteristics

Because of the difficulty in diagnosing LCNEC on small biopsy specimens and cytology samples, most clinical data come from surgical series, which may bias the results (i.e., selection of younger patients with less comorbidity). Reported symptoms at initial presentation often include coughing, weight loss, hemoptysis, chest pain, and fever[76-78]. Classic paraneoplastic syndromes, such as Cushing syndrome seen in SCLC or the carcinoid syndrome rarely associated with lung carcinoids, are seldom diagnosed in LCNEC[79,80].

The incidence of LCNEC is about 3% in reviewed case series of resected pulmonary malignancies[2]. In these LCNEC series, the average age of initial presentation was 64 years (range, 30 to 88 years) with most patients being male (mean, 80%; range, 54% to 89%) and former heavy smokers (89% to 100%) (Table 2.3). Data from two studies have challenged that the incidence of LCNEC is substantially higher in men. In 100 cases of confirmed resected LCNEC, the female: male ratio was 5:9 (46%:54%)[81]. In a study of clinical characteristics extracted from the Surveillance, Epidemiology and End Results (SEER) database, the findings were similar, with a female: male ratio of approximately 5:9 (45%:55%)[82].

Table 2.3 Overview of the clinical characteristics of patients with pulmonary neuroendocrine tumor

	Typical Carcinoid	*Atypical Carcinoid*	*LCNEC*	*SCLC*
Demographics				
Median Age (yrs.)	40-50	50-60	60-70	50-70
Associated with smoking	No	? Yes	Yes	Yes
Male : female ratio	1:1	1:1/2:1	>2.5:1	<2.5:1
Histopathological features				
Mitosis per 10 HPF's	<2	2-10	>10 (median, 70)	>50 (median, 80)
Necrosis	No	Yes (dot like)	Yes (abundant)	Yes (abundant)
Nucleoli	Occasional	Common	Very common	Absent or inconspicuous
Nuclear-to-cytoplasmic ratio	Moderate	Moderate	Low	High
Nuclear chromatin	Finely granular	Finely granular	Usually vesicular, may be finely granular	Finely granular
Shape	Round, oval, spindled	Round, oval, spindled	Round, oval, polygonal	Round, oval, spindled
Imaging				
Central-to-peripheral ratio	3:1	3:1	1:4	10-20:1
Classification/ossification (%)	30	30	9	Up to 23
Stage (%)				
I	87	43	18	3
II	10	29	7	2
III	3	14	24	27
IV	0	14	42	57
Unknown	NA	NA	9	11
Extra thoracic metastases	3%	21%	35%	60-70%
Enhancement	High; central or rim	High; central or rim	High	High with necrosis
FDG uptake on PET	Low	Low/moderate	High	High
SRS uptake (%)	80	80	55	Primary 95, metastasis: 45-60

Abbreviations: HPF's, high power fields; FDG, Fluorodeoxyglucose; PET, positron emission tomography

Staging

No classification staging system currently exists that specifically addresses LCNEC; therefore, the 7th edition of the tumor, node, metastasis (TNM) staging for NSCLC is used for staging LCNEC[83].

Imaging

Computed tomography (CT)

Findings of LCNEC on CT are nonspecific and comparable to that of other solid pulmonary malignancies. LCNEC is predominantly situated in the periphery (67% to 97%)

of the lungs but like SCLC, can also be centrally located (3% to 33%)[76,84,85]. In radiologic and surgical case series, LCNEC had a slight tendency to be situated in the upper lobes. The border of the tumor is usually lobulated, but can be spiculated. On CT evaluation, the average diameter of the primary tumor is approximately 40 mm (range, 7 to 100 mm)[76,84,85]. Necrosis is commonly seen and can present as an inhomogeneous enhancement, especially in larger nodules. Calcification has been reported in 7% to 21% of all primary LCNEC tumors, and it has been hypothesized that these are dystrophic calcifications that can arise in areas of necrosis[76,84-86].

Positron emission tomography

The available data suggest that LCNEC has a high uptake of fluorine 18-fluorodeoxyglucose (FDG) with a mean standardized uptake value (SUV) of 12.0 (range, 3.9 to 25.6)[84,87], comparable with results found in SCLC[88].

Somatostatin receptor scintigraphy

Expression of somatostatin is frequently found in neuroendocrine tumors and is known for its regulation of hormones including glucagon, gastrin, insulin, and the growth hormone. Currently, five somatostatin subtype receptors (SSTR) are known and classified as SSTR-1 through SSTR-5. In a surgical series, the different receptors were found to be present to varying degrees in typical carcinoid, atypical carcinoid, and LCNEC. Except for SSTR-5, there was a tendency toward decreased expression in well to poorly differentiated neuroendocrine carcinomas[89].

Radiographic detection of SSTR in tumors is possible with Indium-111 pentetreotide scintigraphy (octreotide scan). An octreotide scan detects radiolabeled octreotide, a synthetic analogue of somatostatin, which binds with a high affinity to SSTR-2, 3, and 5 after intravenous injection. In a small study evaluating LCNEC preoperatively, 55% of primary lesions (10 of 18) showed activity on octreotide scan[90]. In another study, the use of technetium-99m ethylene diamine-diacetic acid/hydrazinonicotinyl-Tyr3-octeotride (99mTc-TOC) scintigraphy was evaluated for the detection of LCNEC[91]. A high sensitivity was found for primary lesions (100%) and supradiaphragmatic metastases (83%), whereas none of the infradiaphragmatic (adrenal glands) metastases was detected and only 11% of all skeletal metastases were detected. Table 2.3 provides a summary of the clinical, pathologic, and imaging findings in neuroendocrine cancers of the lung.

Therapy

Surgery

Surgical treatment of LCNEC may be indicated for stage I and II disease, like other NSCLC histologic types. However, surgical treatment is rarely an option, as approximately 45% of patients present with metastatic disease (Table 2.3)[82,92]. In addition, evidence regarding surgical treatment for LCNEC is rare because no randomized trials have been conducted on the subject.

In a selection of studies, surgical case series of LCNEC were reported, spanning the period of 1982 through 2010 (Table 2.4). Most of the studies included a pathology review of all resected tissues. Lobectomy was the most frequently used surgical treatment modality (48% to 95% of patients with LCNEC), although several patients received a pneumonectomy or bilobectomy. Systematical node dissection was performed in most cases, and the reported resection status, R0, was 84% to 100%. The stage of disease was recorded according to pTNM staging editions IV to VII. There was no lymph node involvement in 46% to 75% of patients, but metastasis after resection was reported in 0% to 7% of the cases studied. After resection, 14% to 34% of the patients were treated with adjuvant chemotherapy, although majority of the post-surgical data were missing.

The reported 5-year survival of patients with resected LCNEC ranged from 28% to 57%, with an average overall survival rate of 43%; however, it should be considered that the clear majority of the patients had stage I to III disease. Five-year survival of patients ranged from 33% to 67% for those with stage I disease and 23% to 75% with stage II disease. However, any conclusion is limited by the use of different TNM staging systems, and the adoption of adjuvant chemotherapy varied considerably from one study to another. Recurrence of disease was reported in 40% to 62% of patients.

Adjuvant treatment

Evidence regarding beneficial effects of adjuvant chemotherapy and radiotherapy for resected LCNEC is limited. Several retrospective case series and one prospective study have been reported. In this chapter, only studies in which treatment for pure or mixed LCNEC were assessed are reviewed. Studies that include large cell carcinoma with neuroendocrine morphology without neuroendocrine differentiation are not addressed.

In 2006, Iyoda et al. conducted a single-arm, nonrandomized, non-blinded prospective trial of patients with resected LCNEC who were treated with postoperative adjuvant cisplatin and etoposide (two cycles)[93]. Fifteen patients with resected LCNEC (13 with stage I disease and four with stage II or higher), including radical lymph node dissection, were included. The results of the prospective study were compared with the results from a retrospective cohort of 23 patients treated with resected LCNEC without adjuvant chemotherapy; clinical characteristics of the two series were comparable. The authors reported a difference in overall survival, however, with an advantage for the cohort that received adjuvant treatment. The 2- and 5-year overall survival was 88.9% and 88.9%, respectively, in the treatment group compared with 65.2% and 47.4% in the control group.

Rossi et al. examined a cohort of 83 patients with resected LCNEC, including 28 patients who received adjuvant chemotherapy[94]. The chemotherapy consisted of SCLC regimens (13 patients) and NSCLC regimens (15 patients), including cisplatin and gemcitabine, carboplatin and paclitaxel, and cisplatin and vinorelbine. Multivariate regression analysis for survival showed that treatment with an NSCLC adjuvant chemotherapy regimen resulted in a relative risk of 15.52 compared with a SCLC regimen, favoring the SCLC regimen (P=0.0001). Stage and tumor size had a relative risk of 2.31 (P=0.029) and 2.15 (P=0.013), respectively. The chemotherapy regimens were not analytically reported. The results were similar in the study by Tanaka et al. of 63 patients with completely resected (R0) LCNEC. Twenty-three (37%) of 63 patients were treated with induction chemotherapy (three patients) or adjuvant chemotherapy (20 patients). Regimens differed, although all contained a platinum agent (combinations of carboplatin or cisplatin with etoposide, paclitaxel, docetaxel, or vinorelbine). Multivariate analyses showed improved survival for patients treated with adjuvant chemotherapy (hazard ratio, 0.323; P=.037). Pathologic stage (I vs. II/III) was not a predictor for survival (hazard ratio, 0.645; P=0.29). The authors also reported possible chemotherapy resistance in patients with LCNEC that were positive for three neuroendocrine markers (NCAM+, chromogranin A+, and synaptophysin+). In this group of patients with triple-positive LCNEC, chemotherapy did not contribute to increased survival.

Sarkaria et al. evaluated 100 patients with surgically resected LCNEC (and combined LCNEC)[81]. Twenty-four patients received induction chemotherapy with a response rate of 63% (15 with partial response, eight with stable disease, and one with progressive disease); 22 (92%) of the 24 patients received a platinum-based chemotherapy regimen. A total of 25 patients received adjuvant chemotherapy, mostly consisting of platinum-based regimens (80%), and 60% received a platinum-based regimen combined with

etoposide. Adjuvant radiotherapy was administered to 15 patients. Forty-two patients received both induction and adjuvant chemotherapy. In patients with completely resected IB-IIIA disease, treatment with platinum-based chemotherapy correlated with a nonsignificant trend in increased survival. Multivariate analysis for survival was significant for stage (hazard ratio for stage III/IV vs. stage I/II, 2.21; *P*=0.011), gender, and pulmonary comorbidities. In a study by Veronesi et al., in which patients with resected LCNEC were evaluated in a retrospective and multicenter setting, 21 patients received induction chemotherapy; among the 15 patients evaluable for response; the response rate was 80% (one complete response, 11 partial responses, two stable diseases, and one progressive disease)[95]. In addition, several patients received radiotherapy postoperatively. Chemotherapy combined with surgery compared with surgery alone was not a significant predictor in the multivariate analysis for survival (hazard ratio, 0.6; *P*=0.274). Stage of disease, age, and type of surgery were significant independent prognostic factors.

Iyoda et al. found that recurrence of disease occurred less frequently among 30 patients who received platinum-based adjuvant chemotherapy compared with 42 patients who received non–platinum-based adjuvant chemotherapy; disease recurred in 33% of patients who received platinum-based chemotherapy compared with 62% of patients who received nonplatinum-based treatment (*P*=0.017)[96]. However, no information was reported about the type of node dissection, the status of surgical margins (R0, R1, and R2), the exact type of chemotherapy, and the median follow-up of patients.

Preliminary results of neoadjuvant therapy in patients amendable for surgery with LCNEC who had a preoperative octreotide scan have been reported in a single study. All patients with positive findings on the scan received the somatostatin analogue octreotide and several patients also received radiotherapy. The results of this study were promising— a significant survival difference (*P*=0.0007) was found for patients treated with octreotide compared with non-treated patients. Limitations of the study included small sample size (total of 18 patients, 10 of whom were treated) and its retrospective nature[90].

Currently, in two prospective studies, the efficacy of adjuvant chemotherapy for resected LCNEC is being evaluated. The UMIN000001319 trial is a Japanese single-arm, multicenter, nonblinded randomized phase II study in which the effect of four cycles of cisplatin and irinotecan in both LCNEC and limited-disease resected SCLC is being assessed. Preliminary results from this study showed an overall and recurrence-free survival rate at 3 years of 86% and 74%, respectively, for the LCNEC cohort. Patients

were classified as having IA to IIIA disease, and 83% of patients completed chemotherapy[97]. The other prospective study, also from Japan (Trial identifier: UMIN-000010298), is a two-arm, randomized double-blind phase III trial in progress that is comparing adjuvant cisplatin and irinotecan with cisplatin and etoposide for stage I-IIIA resected LCNEC[98].

Therapy for metastatic LCNEC

There is minimal evidence supporting the use of systemic therapy for metastatic LCNEC. As previously mentioned, LCNEC is a rare disease and diagnosis is based on histology from larger surgical biopsies. In metastatic lung cancer, surgical biopsies are scarce. In most studies reporting on advanced LCNEC, the diagnosis is based on a small biopsy sample or resected LCNEC case series containing metastatic recurrence of disease after surgical resection.

At the time of publication, two small single-arm, prospective multicenter phase II studies have been conducted in metastatic LCNEC. Both studies evaluated a SCLC chemotherapy regimen; in a European study of 42 patients, the regimen was cisplatin and etoposide; in a Japanese study of 44 patients, the regimen was cisplatin and irinotecan[99,100]. All patients had stage IIIB or IV disease, were chemotherapy-naïve, and had an Eastern Cooperative Oncology Group performance status of less than 2. A central pathology review was performed in more than 95% of the patients. The diagnosis was revised in 25% of the patients who received cisplatin and etoposide and 28% of the patients who received cisplatin and irinotecan). Most revised diagnoses were SCLC.

In the cisplatin and irinotecan study, patients with confirmed LCNEC had a response rate of 47%; no patients had a complete response, 14 had a partial response, 10 had stable disease, and six patients had progressive disease. The median progression-free survival time was 5.8 months (range, 3.8 to 7.8 months) and median overall survival was 12.6 months (range, 9.3 to 16.0 months). Compared with the 10 patients who were diagnosed with SCLC in this study, patients with LCNEC had a significant shorter overall survival time (12.6 vs. 17.3 months; P=0.047), although median progression-free survival was similar. Thirty patients (65%) completed four cycles of chemotherapy. Second-line chemotherapy consisted mostly of amrubicin (a drug registered only in Japan), platinum-based chemotherapy, and docetaxel. The response rate of second-line chemotherapy was not reported.

In the study of cisplatin and etoposide, patients with confirmed LCNEC had a response rate of 34%: no patients had a complete response, 10 had a partial response, and nine

had stable disease, with progression-free survival of 5.0 months (range, 4.0 to 7.9 months) and overall survival of 8.0 months (range, 3.7 to 7.9 months). In the other histology group, which consisted of nine patients with SCLC, one with atypical carcinoid, and one with neuroendocrine-expressing NSCLC, the progression-free survival was 3.1 months (range, 2.8 to 8.5 months) and overall survival was 7.0 months (range, 3.0 to 9.0 months). There was no significant difference between patients with LCNEC and the other histology group (*P*=0.55). The reported median follow-up was 37.2 months.

The published literature includes several retrospective studies in which NSCLC-based regimens are evaluated; however, only a limited number of cases are reported. Rossi et al. reported on 15 patients with resected LCNEC who were treated with cisplatin and gemcitabine or carboplatin and paclitaxel and gemcitabine monotherapy at the time of disease recurrence[94]. None of the patients who received NSCLC-based therapy had a response, but six patients who received an SCLC regimen had an objective response. Sun et al. performed a study of 45 patients with LCNEC treated with regimens specifically for SCLC or NSCLC[101]. The authors reported a response rate of 73% (eight of 11 patients) in the group treated with SCLC regimens compared with a 50% (17 of 34 patients) treated with NSCLC regimens (*P*=0.19). Platinum-based regimens combined with etoposide or irinotecan yielded a response rate of 73% in the SCLC group and 100% in the NSCLC group. Platinum-based regimens containing gemcitabine resulted in a response in 41% of the patients. In another study, approximately five of seven patients had a response to a combination of a platinum agent and paclitaxel[102].

Available information about second-line chemotherapy for the treatment of LCNEC is almost non-existent. In one retrospective study, a response rate of 23% (three of 13 patients) was documented for second-line amrubicin monotherapy[103]. A single-arm, nonblinded phase II study of bevacizumab and docetaxel for second-line chemotherapy after platinum-based chemotherapy has been initiated in Japan (Trial identifier: UMIN-000011713).

Molecularly targeted therapies

Targeted therapies have yet to be fully investigated for the treatment of LCNEC. In addition, the role of octreotide analogues has not been investigated in LCNEC. There has only been one study in which SSTR-targeted therapies have been described, and prolonged overall survival was reported in patients with SSTR-positive metastatic disease[104].

In a multicenter open-label, single-arm phase II study in Germany that was ongoing at the time of publication, the mTOR inhibitor everolimus is combined with paclitaxel and carboplatin for the treatment of advanced LCNEC. Completion of this study is expected by the end of 2015.

Combined LCNEC

LCNEC can be expressed as a pure form of the disease, but it can also be expressed in combination with other solid tumors. LCNEC is occasionally seen in combination with an adenocarcinoma or squamous cell carcinoma component (Figure 2.3). Other rare forms are combinations of LCNEC and giant cell carcinoma or spindle cell carcinoma. The exact incidence of combined LCNEC is unknown, but it has been reported to be between 6% and 31% in large series of surgically resected LCNEC (Table 2.4)[2,81,105,106]. Now, combined tumors with an LCNEC component should be classified as combined LCNEC and treated as LCNEC, except for LCNEC combined with SCLC, which should be classified as a combined SCLC and treated as SCLC.

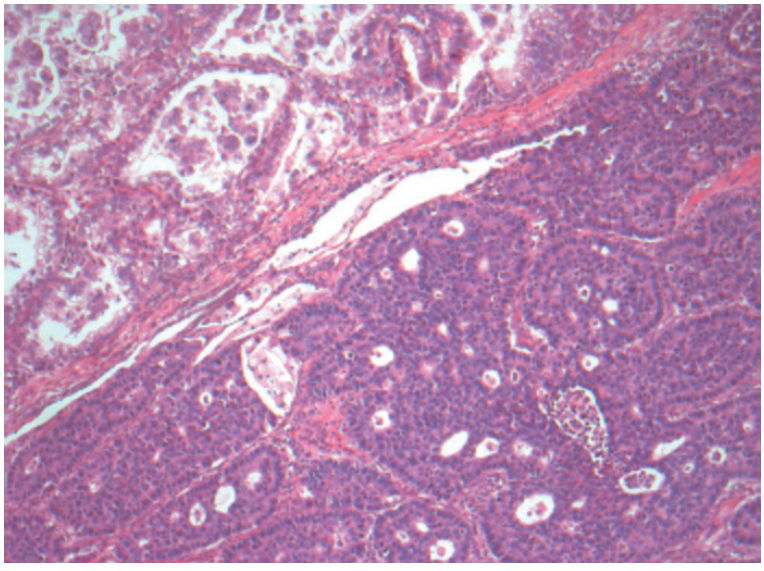

Figure 2.3 Low magnification view of a pulmonary large cell neuroendocrine carcinoma (down right corner) combined with adenocarcinoma (upper left corner) (H&E, x40). (Courtesy of Dr. M. Béndek, Maastricht University Medical Centre.)

Table 2.4 Overview of clinical characteristics from a selected group of large studies on surgical resected LCNEC

	Takei[11]	Rossi[10]	Veronesi[12]	Varlotto[13]	Fournel[14]	Tanaka[15]	Sarkaria[16]	Grand[17]	Grand[17]	Kinoshita[18]	Asamura[19]
Study cohort information											
Time period	82-99	90-04	88-04	01-07	00-10	01-09	92-08	80-09	80-09	95-10	*
Pathology review board	3	3	1 (each	no review	1	1	2	*	*	3	Central
Number of pathologist			centre)								6
Centres	1	2	multi	multi	1	1	1	2	2	1	multi
Histology											
Pure LCNEC (%)	82 (94)	83	144	324	63	63	77 (77)	52		56 (69)	126 (89)
Combined LCNEC (%)	5 (6)						23 (23)		50	25 (31)	15 (11)
Clinical characteristics											
Male (%)	77 (86)	73 (88)	117 (81)	179 (55)	49 (77)	54 (87)	54 (54)	37 (71)	43 (86)	69 (85)	126 (89)
Age, mean	62	*	63	67	64	67	64	60.5	61.4	70	66
Smoker (%)	85 (98)	80 (96)	135 (94)	*	56 (89)	58 (92)	98 (98)	50 (95)	47 (94)	81 (100)	139 (99)
Surgery (%)											
Segment/wedge resection	8 (9)	*	15 (10)	71 (22)	4 (6)	8 (13)	9 (9)	7 (13)	3 (6)	-	*
Lobectomy	61 (70)	*	95 (66)	235 (73)	46 (73)	55 (87)	80 (80)	33 (63)	24 (48)	77 (95)	*
Bilobectomy	6 (7)	*	7 (5)	*	1 (1)	0	0	2 (4)	3 (6)	-	*
Pneumonectomy	12 (14)	*	24 (17)	18 (6)	12 (19)	0	11 (11)	10 (19)	20 (40)	4 (5)	*
SND, +=yes, (%)	60 (69)	+	138 (94)	*	+	*	*	+	+	+	*
Resection status R0 (%)	*	*	136(94)	*	63 (100)	63 (100)	90 (90)	50 (96)	43 (86)	*	*
Lymph node status (%)											
N0	43 (49)	62 (75)	*	224 (69)	29 (46)	*	65 (65)	27 (52)	29 (58)	*	76 (54)
N1	17 (20)**	21 (25)**	*	55 (17)	15 (24)	*	15 (15)	10 (19)	11 (22)	*	26 (18)
>N2	17 (20)**	21 (25)**	*	39 (12)	19 (30)	*	23 (23)	15 (29)	10 (20)	*	36 (26)
Stadium (%) ([TNM 4-7])											
I	41 (47)[4]	54 (66)[6]	73 (51)[*]	186 (57)[*]	22 (35)[7]	24 (45)[6]	44 (44)[7]	20 (38)[7]	20 (40)[7]	48 (59)[7]	63 (45)[5]
II	13 (15)[4]	16 (20)[6]	29 (20)[*]	53 (16)[*]	16 (25)[7]	16 (26)[6]	27 (27)[7]	13 (25)[7]	14 (28)[7]	17 (21)[7]	22 (16)[5]
III	30 (34)[4]	13 (14)[6]	40 (28)[*]	59 (18)[*]	25 (40)[7]	12 (19)[6]	25 (25)[7]	17 (33)[7]	13 (26)[7]	15 (19)[7]	45 (32)[5]
IV	3 (3)[4]	0[6]	2 (1)[*]	0[*]	0[7]	0[6]	4 (4)[7]	1 (2)[7]	2 (4)[7]	1 (1)[7]	10 (7)[5]
Adjuvant chemotherapy (%)											
N (+=yes)	12 (14)	28 (34)	24 (17)	*	+ (*)	20 (32)	25 (25)	*	*	+ (*)	*

Table 2.4 (continued)

	Takei[11]	Rossi[10]	Veronesi[12]	Varlotto[13]	Fournel[14]	Tanaka[15]	Sarkaria[16]	Grand[17]	Grand[17]	Kinoshita[18]	Asamura[19]
Survival and recurrence											
Survival (%)											
5 year	57	27.6	43	41[ii]	49.2	44.9	*	39	38	53.3	40.3
Survival, stadium 5 year (%)											
I	67	33	52	60 [ii]	*	*	53	*	*	*	58
II	75	23	59	*	*	*	61	*	*	*	32
III	45	8	20	*	*	*	24[i]	*	*	*	*
IV	0	-	-	-	*	*	24[i]	*	*	*	*
Median follow-up, months	*	17	27	15	*	32.3	34	73[iii]	73[iii]	60	60
Recurrence (%)	32 (39)	54 (62)	58 (40)	*	*	*	38 (38)	*	*	*	68 (48)

*Data not reported in article. ** Lymph node status N1+N2. [i] Stadium III and IV disease combined. [ii] Calculated for 4 years of survival. [iii] Reported mean value

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SND, Systematic nodal dissection

Prognosis

LCNEC

Asamura et al. reported that the survival curve for LCNEC is superimposable to that for SCLC[106]. Depending on the series, 5-year survival rates ranged from 33% to 62% for stage I, 18% to 75% for stage II, 8% to 45% for stage III, and 0% for stage IV[93,94,106,107].

Conclusion

Pulmonary neuroendocrine tumors are a class of tumors likely arising from the neuroendocrine cells of the bronchopulmonary epithelium. The behavior of pulmonary neuroendocrine tumors varies with their degree of differentiation: typical carcinoids have a more indolent behavior, rarely metastasizing; atypical carcinoids that are intermediate grade have an increased tendency to spread systemically; and LCNECs are high grade, with an aggressive phenotype like that in SCLC. Although metastatic LCNEC resembles SCLC in clinical behavior, the optimal chemotherapy regimen is not clear in this setting.

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Chapter 3

Clinical features of large cell neuroendocrine carcinoma: a population based overview

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Abstract

Pulmonary large cell neuroendocrine carcinoma (LCNEC) is an orphan disease and few data are available on clinical characteristics. Therefore we analyzed LCNEC registered in the Netherlands Cancer Registry (NCR) and compared data with small cell (SCLC), squamous cell (SqCC) and adenocarcinomas (AdC).

Histologically confirmed LCNEC (*N*=952), SCLC (11,844), SqCC (19,633) and AdC (24,253) cases were selected from the NCR (2003-2012). Patient characteristics, metastasis at diagnosis (≥2006), overall survival (OS) including multivariate COX models, and first-line treatment were compared for stage I-II, III and IV disease.

LCNEC increased from 56 patients in 2003 to 143 in 2012, attributing 0.9% of all lung cancers. Stage IV LCNEC patients (*N*=383) commonly had metastasis in liver (47%), bone (32%) and brain (23%), resembling SCLC. Median OS (95% confidence interval) of stage I-II, III and IV LCNEC was 32.4 (22.0-42.9), 12.6 (10.3-15.0) and 4.0 (3.5-4.6) months, respectively. Multivariate adjusted OS of LCNEC resembled SCLC and was poorer than SqCC and AdC. However, frequency of surgical resection and adjuvant chemotherapy resembled SqCC and AdC more than SCLC

Diagnosis of LCNEC increased in recent years. Metastatic pattern of LCNEC resembles SCLC as does OS. However, early stage treatment strategies seem more comparable to SqCC and AdC.

Introduction

Large cell neuroendocrine carcinoma (LCNEC) is a high-grade carcinoma that expresses a neuroendocrine growth pattern (i.e. organoid, nesting, trabeculae, palisading cells or rosettes) and immunohistochemical neuroendocrine differentiation. In the third World Health Organization classification (2004), LCNEC was a subcluster of large cell carcinoma (LCC) and considered as part of the pulmonary neuroendocrine tumor (NET) spectrum. In the fourth WHO classification (2015), the diagnostic criteria for LCNEC were reproduced from those proposed in 1999 and LCNEC was moved from the LCC chapter to the NET chapter[1,2]. Previous studies have shown that incidence of LCNEC is low, with a reported rate of 3% in surgically resected case series[3]. Nevertheless, according to the United States Cancer Registry (SEER) (2003–2007) and the Netherlands Cancer Registry (NCR) (1990–2010), pulmonary LCNEC incidence is
Rising[4,5].

Because LCNEC expresses neuroendocrine features, it is suggested to treat LCNEC as small cell lung cancer (SCLC)[6,7]. However, to date, no randomized trials investigating optimal treatment of LCNEC have been performed. Furthermore, it is unclear whether the LCNEC clinical presentation resembles non-small cell lung cancer (NSCLC) or SCLC. Recently, in the SEER registry, clinical characteristics of LCNEC (n=1211), SCLC (n=33 304) and LCC (n=8295) were compared. It was shown that compared to SCLC, LCNEC was more often diagnosed in an early stage and subsequently surgically treated, although data regarding adjuvant chemotherapy or specific stage III–IV disease treatment were not reported, as chemotherapy was not registered by SEER.

Series of surgically resected stage I–III lung cancer have shown that LCNEC and SCLC prognosis are similar[8,9]. However, the SEER registry concluded that the prognosis of resected early-stage LCNEC resembled that of LCC and was superior to SCLC [4]. Two small studies in advanced LCNEC (n=25 and n=14) reported that overall survival (OS) of LCNEC was similar to that of SCLC[10,11]. In one European phase II trial, OS of advanced LCNEC patients (n=29) was similar to results found in trials of advanced SCLC, while the response to chemotherapy was inferior for LCNEC compared to SCLC in a Japanese phase II trial (n=30)[12,13].

Thus, from currently available data, it is not clear whether the LCNEC clinical presentation resembles SCLC or NSCLC. In addition, optimal treatment of LCNEC is not defined by current guidelines. The aim of this study was to evaluate the clinical presentation, prognosis and currently applied treatment of LCNEC in comparison to

other lung cancer subtypes. Therefore, we analyzed data from all histologically diagnosed LCNEC patients entered in the NCR, and compared those with both SCLC and NSCLC.

Patients and methods

Data sources

For this retrospective population-based study, data for patients diagnosed between 2003 and 2012 were obtained from the NCR. The registry has a nationwide coverage with >95% completeness of case ascertainment and patient data are collected in a standardized manner[14]. Furthermore, patients' records are linked to the Netherlands Pathology Registry and Centralized Civil Registry for pathology confirmation and annual vital status update.

Available data were year of diagnosis, histology (based on the International Classifications of Disease–Oncology (ICD-O), Third Edition), tumor grade, tumor–node–metastasis (TNM) classification (2010 or later: according to the TNM-7; 2009 or earlier: according to the TNM-6 classification) (generally, as clinical (c)TNM was overruled by pathological (p)TNM; in cases with neoadjuvant chemotherapy, cTNM overruled pTNM), first-line treatment modality and time from diagnosis till death or last follow-up. Metastatic sites at diagnosis (i.e. before treatment) were collected from documented clinical data (cTNM and/or pTNM) with a maximum of three separate locations. Subsequently, sites of metastases were combined into organ-specific subcodes (e.g. femur and rib into "bone").

Study population

NCR data were retrieved on March 21, 2014, and included all patients with a histologically confirmed diagnosis of LCNEC (ICD-O code 8013), SCLC (8041–8043), SqCC (8050–8084) or AdC (8140–8230, 8250–8550 or 8570–8574), diagnosed between January 1, 2003, and December 31, 2012. AdC cases with neuroendocrine differentiation (ICD-O-3 code 8574) were clustered into AdC to prevent inclusion of tumors with neuroendocrine differentiation but without neuroendocrine morphology into the LCNEC cohort. LCNEC cases classified as grade I–II tumors were not included to avoid possible contamination with carcinoid tumors. In addition, LCC (not otherwise specified) was omitted as recent evidence suggests that up to 80% can be classified as SqCC or AdC upon revision in conjunction with immunohistochemical and molecular profiling[15,16].

Other exclusion criteria were no recorded TNM classification, metachronous lung cancer or incomplete survival data.

To evaluate the clinical characteristics, metastatic pattern, first-line treatment and OS, several subcohorts were composed: all stages, stage I–II, stage III and stage IV disease. Additionally, first line treatment was compared separately for patients entered from 2010 to 2012 in order to observe possible temporal and TNM-6 to TNM-7 transition effects. For metastatic pattern analysis, additional exclusion criteria were applied: diagnosis before 2006 (since 2006, the metastasis locations were recorded systematically (≥97%)), no documentation of metastatic sites, previous malignancy diagnosed within 5 years of lung cancer diagnoses and patients with stage IV disease classified according to TNM-6 solely based on pulmonary metastases (possibly T4 in TNM-7, i.e. no stage IV disease). Finally, prevalence of pleural metastasis was only analyzed in patients classified according to TNM-7 to prevent underrating, as TNM-6 recorded pleural metastasis as IIIB disease and it was not feasible to reclassify this patient group.
This study was approved by the data monitoring committee and the medical ethical board of Maastricht University Medical Center (Maastricht, The Netherlands). Analyses were performed according to NCR guidelines and national privacy regulations.

Statistical analysis

Incidence of LCNEC was calculated as fraction of the reported lung cancer incidence[17]. The Chi-squared and Fisher's exact test were used to compare categorical data and confidence intervals of proportions were calculated with the Wald (asymptotic) method. Medians of continuous variables were compared with a Mann–Whitney U-test. Censoring took place at the closing date (December 31, 2012) or at the last date of follow-up if patients emigrated. OS was calculated according to the Kaplan–Meier method and tested with the log-rank test. To examine effects of histology on survival, several stratified multivariate Cox regression analysis models were constructed including the covariates age, sex, histology, TNM (7 versus 6), N stage and T stage, and depending on the stage, treatment was included. Assumptions of proportional hazards were investigated by visual inspection of the complementary log–log plots. In cases where a hazard ratio (HR) was nonproportional, time-dependent HRs were reported at the cut-off point where nonproportionality started to influence the results. Two sided *P*-values <0.05 were considered significant. Analyses were performed using SPSS (version 22; IBM, Armonk, NY, USA).

Results

Between 2003 and 2012, 59 283 patients with LCNEC, SCLC, SqCC or AdC were entered in the NCR, of whom 56 682 patients were eligible for all-stage analysis (Table 3.1) and 16 537 patients for metastatic site analysis (CONSORT flow diagram in Figure 3.1). 999 (1.7%) out of 59 283 histologically selected patients were diagnosed with LCNEC, of whom 952 were eligible for the study. The total incidence of LCNEC as proportion of all lung cancers was 0.9%. Annual occurrence of LCNEC increased by 255% from 56 cases in 2003 to 143 in 2012 (Figure 3.2a and 3.2b) with the sharpest increase in 2008. The percentage of LCNEC diagnosed in stage IV disease increased significantly over time: from 45.0% (n=144) in 2003–2007 to 58.5% (n=370) from 2008 onwards (*P*<0.001) (Figure 3.2c).

Table 3.1 Baseline characteristics according to morphological subtype

Variable	Histology										
	LCNEC (N=952)		SCLC (N=11,844)		SqCC (N=19,633)		AdC (N=24,253)		SCLC	SqCC	AdC
	No.	%	No.	%	No.	%	No.	%	*p*-values versus LCNEC		
Age											
Mean(SD)	65.5 (10.5)		66.7 (9.7)		68.8 (9.4)		64.6 (10.7)				
Median	66		67		70		65		0.14*	<0.001*	<0.001*
IQR	52-80		53-81		57-83		49-81				
Gender									0.01	<0.001	<0.001
Male	595	62.5	6903	58.3	15055	76.7	13404	55.3			
Female	357	37.5	4941	41.7	4578	23.3	10849	44.7			
TNM Stage									<0.001	<0.001	<0.001
I	162	17.0	370	3.1	4745	24.2	5318	21.9			
II	90	9.5	223	1.9	2497	12.7	1705	7.0			
III	186	19.5	3389	28.6	7009	35.7	5134	21.2			
IV	514	54.0	7862	66.4	5382	27.4	12096	49.9			
Tumor stage									<0.001	<0.001	0.002
T1	183	19.2	879	7.4	2665	13.6	5394	22.2			
T2	297	31.2	3234	27.3	7886	40.2	7903	32.6			
T3	118	12.4	1077	9.1	2890	14.7	2570	10.6			
T4	266	27.9	4933	41.6	5607	28.6	6650	27.4			
Tx	88	9.2	1721	14.6	585	2.9	1736	7.2			
Nodal stage									<0.001	<0.001	0.03
N0	359	37.7	2042	17.2	8616	43.9	10227	42.2			
N1	96	10.1	509	4.3	2289	11.7	2033	8.4			
N2	314	33.0	6023	50.9	6478	33.0	7645	31.5			
N3	183	19.2	3270	27.6	2250	11.5	4348	17.9			

*Mann-Whitney *U* test

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma; SD, standard deviation; IQR, inter quartile range

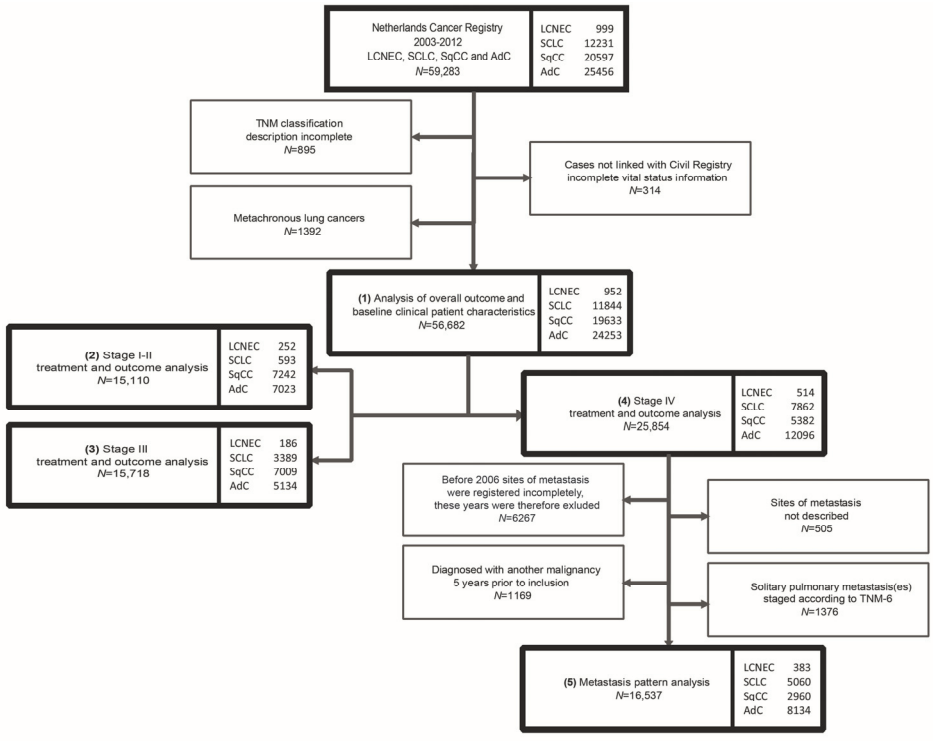

Figure 3.1 A CONSORT diagram is presented that describes the selection of cases from the Netherlands Cancer Registry. Patients with histo-pathological diagnosed lung cancer during 2003-2012 were included if diagnosis was according to one of the following morphology codes: LCNEC (8013), SCLC (8041-8043), SqCC (8050-8084) and AdC (8140-8230, 8250-8550, 8570-8574)
Abbreviations: TNM, tumor-node-metastasis classification; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma

Differences in baseline clinical characteristics

Baseline clinical characteristics are presented in Table 3.1. In LCNEC, 63% were male and the mean±SD age was 65.5±10.5 years. Compared to LCNEC, the stage distribution of SCLC was more advanced and SqCC was more frequent diagnosed as early disease. With the exception of a lower percentage of stage I disease, LCNEC stage distribution was comparable to AdC. Nodal stage (N)2–N3 disease was present in 52% of LCNEC, 79% of SCLC (P<0.001), 45% of SqCC (P<0.001) and 49% of AdC (P=0.10). In stage IV patients, the incidence of N2–N3 disease was significantly lower in LCNEC (69%) than in SCLC (80%) (P<0.001) and was comparable to SqCC (66%, P=0.30) and AdC (66%, P=0.34).

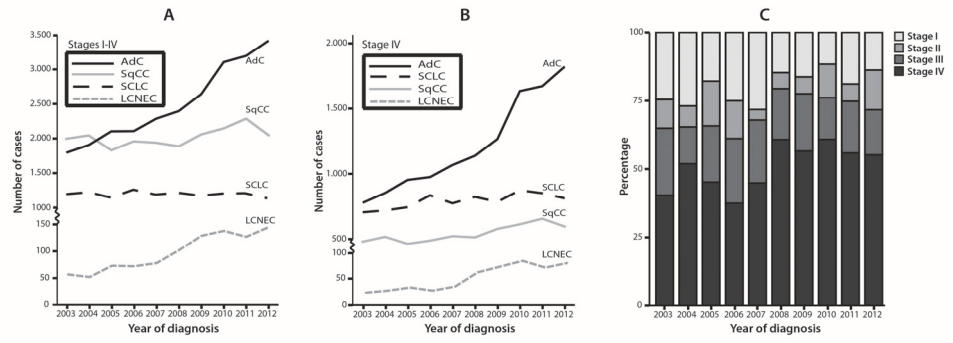

Figure 3.2 Incidence of histo-pathological diagnosed cases of lung cancer registered in the Netherlands Cancer Registry between 2003 and 2012. **A**) Trend in frequency of individual morphological subtypes. **B**) Tend in frequency of individual morphological subtypes with stage IV. **C**) Trend in stage distribution of LCNEC between 2003 and 2012
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma

Prevalence of organ specific metastasis at diagnosis

LCNEC metastasis occurred in liver (47%), bone (32%), brain (23%), adrenal gland (19%), lung (14%), pleura (7%) and extra thoracic lymph nodes (16%) (Figure 3.3). LCNEC had significantly fewer liver and more brain metastasis than SCLC. The prevalence of metastasis was not statistically different in the other organs. Patients with LCNEC had significantly fewer pleural and lung metastasis but more frequent liver and extra thoracic lymph node metastasis than patients with SqCC and AdC. Patients with LCNEC had more brain metastasis than those with SqCC, which was similar in AdC, whereas bone metastasis in LCNEC occurred less commonly than in patients with AdC.

Comparison of outcome

The median (range) follow-up of the whole cohort was 52 (0–120) months. Median OS (95% CI) of LCNEC was 8.7 (7.9–9.6), SCLC 7.1 (6.9–7.3), SqCC 13.1 (12.7–13.4) and AdC 11.8 (11.5–12.2) months, respectively (Figure 3.4). Median OS of stage I–II, III and IV LCNEC was 32.4 (22.0–42.9), 12.6 (10.3–15.0) and 4.0 (3.5–4.6) months, respectively, and that of stage IV chemotherapy-treated LCNEC was 7.7 (6.8–8.6) months. Table 3.2 depicts the models used for multivariate analysis of OS. Nonproportionality was observed for the covariate histology in stage I–II (Figure 3.4b) and, therefore, HRs were calculated separately (i.e. HR for LCNEC differed between time period of <10 months and ≥10 months). Patient variable adjusted OS of stage I–II LCNEC was superior to that of SCLC (<10 months and ≥10 months: HR (95% CI) 1.85 (1.27–2.69) and 1.56 (1.21–2.00),

respectively). Compared to LCNEC, OS of SqCC and AdC was only significantly better after 10 months (HR 0.65 (0.52–0.80) and 0.64 (0.52–0.80)). A separate model analyzing patients treated with surgery showed no statistical difference between LCNEC and SCLC, yet the OS of SqCC and AdC showed similar results to the overall model. In stage III disease, the adjusted model showed no statistically significant difference between LCNEC and SCLC, SqCC or AdC. In stage IV, the adjusted model revealed that the OS of LCNEC was worse than that of SCLC (HR 0.87 (0.79–0.95)), SqCC (HR 0.79 (0.72–0.87)) and AdC (HR 0.79 (0.72–0.86)). However, in patients treated with chemotherapy, the OS of LCNEC was similar to that of SCLC while the OS of SqCC and AdC remained significantly longer.

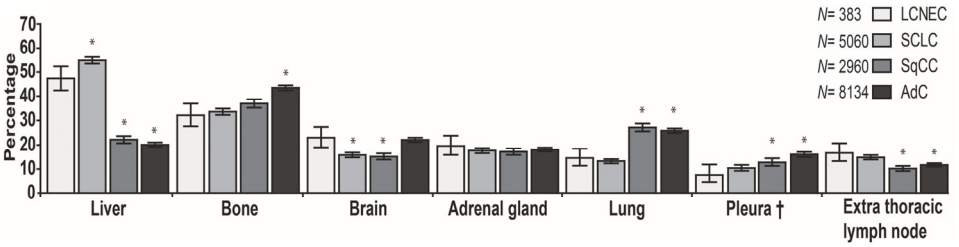

Figure 3.3 Prevalence of sites of metastases at primary diagnosis of lung cancer recorded between 2006 and 2012. Metastatic sites are clustered into organ specific locations and analyzed for each lung cancer subtype. All subtypes are compared with LCNEC. †: Analyzed only in TNM-7; * Significant, *P*<0.05 Chi-square test
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma.

Comparison of first-line treatments

Stage I–II LCNEC was generally treated with surgical resection (87.3%), as was AdC (*P*=0.09), but fewer resections were performed in SCLC (*P*<0.001) and SqCC (*P*<0.001) (Table 3.3). Adjuvant chemotherapy was administered to 23.2% of LCNEC patients, which was less than in SCLC (*P*<0.001) but more frequent than in SqCC (*P*<0.001) and AdC (*P*<0.001). Patients with stage III LCNEC were treated with a combination of chemotherapy and radiotherapy (30.6%), surgical resection (21.0%), chemotherapy (14.5%) or received no treatment (19.4%). Treatment practice differed significantly from SCLC (*P*<0.001) but was comparable to SqCC (*P*=0.15) and AdC (*P*=0.10). In stage IV, 45.7% of LCNEC patients received no treatment, which was more frequent than in SCLC and AdC (*P*<0.001), and comparable to SqCC (*P*=0.76). Chemotherapy was administered in 38.1% of LCNEC patients. This was significantly less than in SCLC (*P*<0.001), more than in SqCC (*P*<0.001) and was equal to AdC (*P*=0.65). To explore whether the time period

affected first-line treatment, the time period of 2010–2012 was analyzed separately and compared to 2003–2009 but reported trends remained consistent (data not shown).

Table 3.2 Univariate and multivariate analysis of overall survival for LCNEC compared to SCLC, SqCC and AdC

		Histology						
		LCNEC	SCLC		SqCC		AdC	
Stage comparison	Variable	HR	HR	95% CI	HR	95% CI	HR	95% CI
Stage I-II	Unadjusted							
	< 10 months*	1	2.16	1.49-3.15	1.43	1.01-2.02	0.76	0.54-1.08
	≥ 10 months*	1	1.62	1.27-2.08	0.75	0.60-0.94	0.56	0.45-0.70
	Adjusted (1)							
	< 10 months*	1	1.85	1.27-2.69	1.15	0.82-1.63	0.84	0.59-1.19
	≥ 10 months*	1	1.56	1.21-2.00	0.65	0.52-0.80	0.64	0.52-0.80
Stage I-II	Adjusted (2)							
surgical cohort	< 10 months*	1	0.71	0.33-1.50	0.72	0.49-1.05	0.56	0.38-0.82
	≥ 10 months*	1	1.14	0.77-1.69	0.47	0.37-0.60	0.52	0.41-0.66
Stage III†	Unadjusted	1	1.04	0.88-1.23	1.00	0.84-1.18	0.83	0.70-0.98
	Adjusted (1)	1	0.93	0.78-1.10	0.88	0.74-1.04	0.86	0.73-1.02
Stage IV	Unadjusted	1	0.94	0.86-1.04	0.88	0.80-0.97	0.76	0.70-0.84
	Adjusted (3)	1	0.87	0.79-0.95	0.79	0.72-0.87	0.79	0.72-0.86
Stage IV *chemotherapy cohort*	Adjusted (3)	1	1.06	0.91-1.23	0.85	0.73-0.99	0.85	0.73-0.99

*Time stratification used to counter non-proportionality (occurring for stage I-II). † Insufficient patients with LCNEC therapeutically treated (e.g. with chemo-radiotherapy or chemotherapy) to allow controlling for treatment

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma; HR, Hazard-ratio; CI, confidence interval; TNM, Tumor-Node-Metastasis classification. 1 age, sex, TNM edition, T stage, N stage; 2 age, sex, TNM edition, T stage, N stage, adjuvant chemotherapy; 3 age, sex, TNM edition, T stage, N stage

Discussion

In this population-based study we confirmed that LCNEC is a rare disease with an average incidence of 0.9% of lung cancer over a 10-year period, while occurrence increased 2.5-fold. The presented results indicate that LCNEC is a highly aggressive form of lung cancer like SCLC, with a poor prognosis in all stages of disease. Nevertheless, there were important differences from SCLC: in stage IV, the prognosis of LCNEC was lower than SCLC, yet similar in selected cases treated with chemotherapy, as well as the clinical presentation, such as the lower proportion of patients with mediastinal lymph node involvement and the currently applied treatment in early disease differed from SCLC.

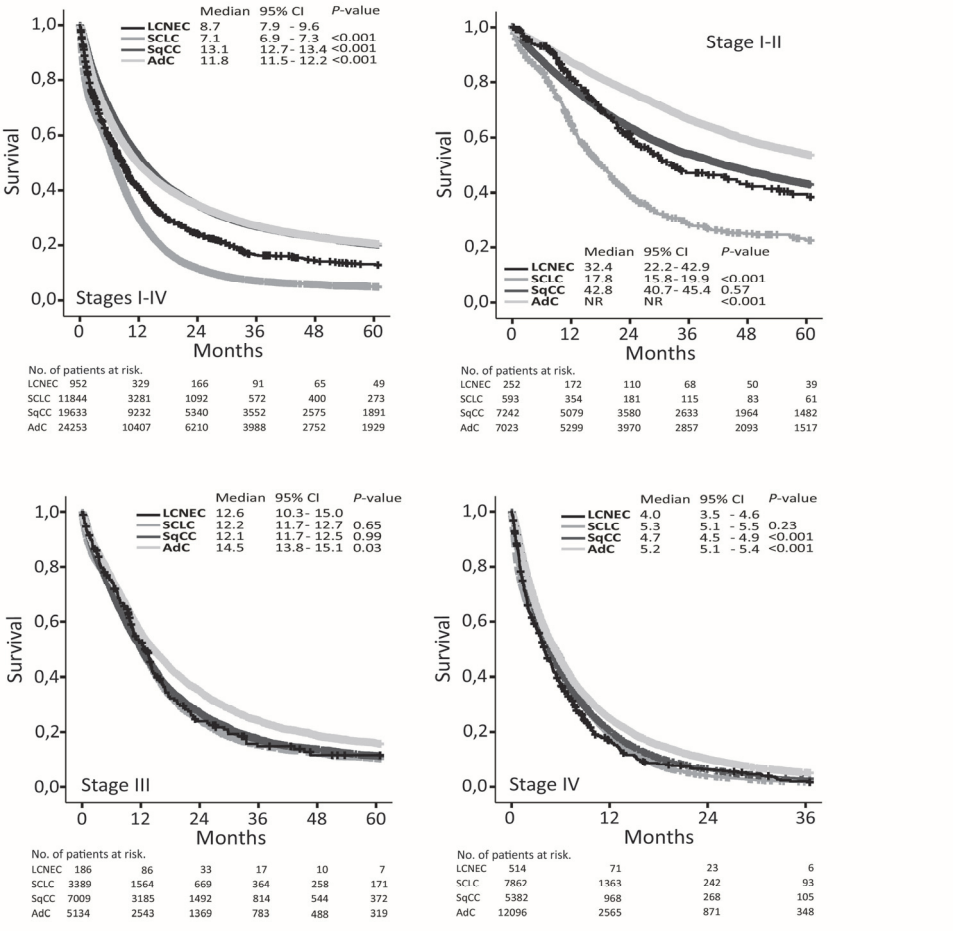

Figure 3.4 Survival curves for LCNEC compared to SCLC, SqCC and AdC in all stages (I-IV) and for stage I-II, III and IV separately.
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma; NR, not reached.

The total incidence of LCNEC (0.9%) as fraction of all diagnosed lung cancer was lower than that reported by institutional surgical series (1.7–3.0%)[3,7] but higher than that reported by the population based SEER registry (0.6%). Probably this is caused by differences in analyzed time period (i.e. 2003–2007). Overall, lung cancer occurrence increased with 24% in the Netherlands (2003–2012), mainly attributed to the increase in AdC[17]. The increase observed in LCNEC, particularly occurring after 2008, might be explained by growing awareness among pathologists of this relatively new entity,

although at that time, no new pathology guideline was published. Additionally, an increased use of immunohistochemical neuroendocrine markers (CD56, synaptophysin and chromogranin-A) in routine diagnostics and an increase in core/needle-biopsy sampling in order to obtain sufficient material for molecular testing (i.e. more tissue) have improved LCNEC diagnosis. In addition, indiscriminate use of markers with low specificity for neuroendocrine differentiation (i.e. CD56 or NSE) could have increased the diagnostic frequency on biopsy tissue specimens. Finally, introduction of the IASLC guideline for diagnosis of lung cancer on biopsies (2011) may have increased awareness of LCNEC diagnosis on biopsies. Currently, LCNEC on small biopsies is referred to as NSCLC, possibly LCNEC when a neuroendocrine morphology and neuroendocrine immunohistochemical staining is confirmed in a biopsy specimen[2,18].

Table 3.3 First-line treatment according to morphological subtype

Variable	Histology										
	LCNEC (N=952)		SCLC (N=11,844)		SqCC (N=19,633)		AdC (N=24,253)		SCLC	SqCC	AdC
	No.	%	No.	%	No.	%	No.	%	*P*-values versus LCNEC		
Treatment in stage I-II									<0.01	<0.01	0.441
No treatment	4	1.6	68	11.5	564	7.8	254	3.6			
Resection	220	87.3	114	19.2	5039	69.9	5816	82.8			
RT	17	6.7	26	4.4	1008	13.9	601	8.6			
CT & RT	4	1.6	270	45.5	288	4.0	109	1.6			
CT	2	0.8	94	15.9	111	1.5	55	0.8			
Other	5	2.0	21	3.5	232	3.2	188	2.7			
Stage I-II resections									<0.01	<0.01	<0.01
Adjuvant CT	51	23.2	91	75.4	769	15.3	786	13.5			
Treatment in stage III									<0.01	0.15	0.10
No treatment	36	19.4	473	14.0	1346	19.2	801	15.6			
Resection	39	21.0	32	0.9	1066	15.2	1254	24.4			
RT	20	10.8	55	1.6	1120	16.0	362	7.1			
CT & RT	57	30.6	1794	52.9	2252	32.1	1492	29.1			
CT	27	14.5	912	26.9	888	12.7	879	17.1			
Other	7	3.8	123	3.6	337	4.8	346	6.7			
Stage III resections									0.02	0.43	0.75
(neo) adjuvant CT	21	53.8	26	81.3	508	47.7	707	56.4			
Treatment in stage IV									<0.01	<0.01	<0.01
No treatment	235	45.7	2147	27.3	2422	45.0	4823	39.9			
Resection	17	3.3	7	0.1	131	2.4	363	3.0			
RT	30	5.8	49	0.6	523	9.7	435	3.6			
CT & RT	22	4.3	447	5.7	325	6.0	436	3.6			
CT	196	38.1	4941	62.8	1744	32.4	4735	39.1	<0.01	<0.01	0.65
Other*	14	2.7	271	3.4	237	4.4	1304	10.8			

* Including targeted treatment (e.g. tyrosine kinase inhibitor)

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma; RT, radiotherapy; CT, chemotherapy

The clinical characteristics of LCNEC corresponded to that of SqCC and AdC closely in the early stage, whereas LCNEC overlapped with SCLC in metastatic disease. Not only this study but also the SEER registry reported that the stage distribution of LCNEC resembled NSCLC[4]. A possible explanation is that advanced LCNEC was underrepresented (e.g. pathologists recognize LCNEC in surgically resected tissue more easily and may overlook LCNEC when assessing biopsies). However, we also found that LCNEC patients presented less often with stage N2–N3 compared to SCLC, and this matched SqCC and AdC, also in stage IV disease. Therefore, it might well be that early-onset LCNEC does not metastasize as rapidly as SCLC, increasing the chance of diagnosis at an earlier stage, an observation that was recently also seen in genetically engineered mouse models[19]. Nonetheless, whenever LCNEC has metastasized, the metastatic pattern resembles SCLC. Unfortunately, the metastatic pattern could not be confirmed in other studies as numbers of included patients (n=22–86) were too small[3,7,20,21].

Conflicting data are available in the literature with regard to prognosis of LCNEC. The OS of LCNEC in surgical case series was similar to that of SCLC[8,9,22], while the SEER registry showed that OS in very early (T1N0M0) LCNEC was comparable to LCC and better than SCLC[4]. Resembling the SEER results, we observed a clear OS difference between LCNEC and SCLC in early-stage disease, but whenever SCLC was surgically treated, OS resembled LCNEC. The prognostic results in stage IV chemotherapy-treated patients were in line with two small cohorts that compared OS of LCNEC and SCLC[10,11] and overall OS of chemotherapy-treated LCNEC resembled OS of the European phase II trial[12].

At present, there are no guidelines that aid physicians in treating LCNEC but we have shown that over recent years, treatment corresponded to SqCC and AdC more closely than to SCLC. The difference in (adjuvant) chemotherapy treatment between LCNEC and SCLC in stage I–II and IV was considerable. Indeed, if LCNEC is considered equally aggressive as SCLC, one would expect the ratio of chemotherapy-treated patients to be similar. Moreover, in the adjusted multivariate Cox regression analysis, the prognosis of LCNEC was poorer than that of SCLC. After selection of chemotherapy-treated patients, the adjusted multivariate Cox regression showed a nonsignificant difference. This increase in prognosis might be an important sign of possible under treatment of patients with stage IV LCNEC disease in the overall population, but requires further investigation.

Because of the rarity of LCNEC, the majority of data comes from single-center, retrospectively diagnosed LCNEC series. In this study, for the first time, we describe the clinical manifestation and treatment of both early- and advanced-disease LCNEC in a large, population-based, histologically diagnosed cohort. Moreover, we were able to

comprehensively define the metastatic pattern of LCNEC at diagnosis and to compare this with SCLC, SqCC and AdC. By doing this, we excluded possible interference from treatment, and by excluding patients with previous malignancies, we minimized confounding from other cancers.

The current study has several limitations. Although only histology-selected cases were included, it remains possible that several tumors diagnosed as LCNEC in this registry were incorrectly classified. In clinical trials, up to 25–27% of LCNEC diagnosed on biopsies was reclassified into NSCLC or SCLC after central revision[12,13]. Furthermore, interobserver studies show poor agreement, underscoring the difficulty in delineating LCNEC from SCLC and NSCLC[23]. However, this population-based study mirrors daily practice. A consequence of the histological selection criteria is the observed relatively high fraction of early-stage NSCLC. Another limitation might be related to the sensitivity of the metastatic pattern analysis as registration of used modalities for diagnostic imaging was not mandatory. Finally, we were not able to transcribe TNM-6 into TNM-7, had no data on smoking status, and were not able to adjust for possible prognostic confounders such as comorbidities, performance score and weight loss, as these variables were not or insufficiently registered in the NCR.

In summary, LCNEC is increasingly encountered, especially in stage IV disease. We have shown that LCNEC in clinical practice is a different entity, resembling SqCC and AdC in early-stage disease but with a comparably poor prognosis and metastatic pattern to SCLC. Nonetheless, possible biological important differences were present, as LCNEC showed less lymphatic N2–N3 pattern than SCLC. In the near future, it is expected that the visibility of LCNEC will increase even more for physicians due to the separate mention of LCNEC in the pulmonary NET chapter of the fourth WHO classification and the recent introduction of criteria for the diagnosis "NSCLC, possible LCNEC" on biopsy specimens[2,18]. Therefore, collaboratively structured international phase III trials are needed to investigate the role of adjuvant chemotherapy in early-stage disease and optimal disease management of advanced-stage LCNEC. Eventually, this research can lead to establishment of broadly accepted guidelines for the treatment of LCNEC.

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Chapter 4

Neuro-endocrine cancer of the lung: a diagnostic puzzle

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Case report

Here we report the case of a pulmonary neuroendocrine tumor (pNET) in which the pathological diagnosis was revised several times over the course of the patient's disease because of atypical behavior of the tumor; consequently, the patient was treated with various treatment schedules.

A 51-year-old female former smoker was referred to our academic hospital with a metastasis of a poorly differentiated carcinoma in a subcutaneous lesion on the posterior thoracic wall (Figures 4.1A–1E; Table 4.1, report I). Revision of the initial biopsy and a new fine-needle aspiration biopsy of an additional subcutaneous mass in the breast revealed a non–small cell lung carcinoma with preference for a large cell neuroendocrine carcinoma (LCNEC) and mitotic index of 19 in 10 high-power fields (HPFs) that most likely originated from the lung (Table 4.1, reports II and III). Both an 18F-fluorodeoxyglucose–positron emission tomography scan and a 68Ga-DOTATATE positron emission tomography/computed tomography scan showed multiple nodules in the right upper and lower lobe as well as subcutaneous and liver metastases. Stage IV LCNEC was diagnosed, and the patient was enrolled in a clinical trial with paclitaxel-carboplatin and bevacizumab with the addition of a nitroglycerine patch (Paclitaxel-Carboplatin-Bevacizumab ± Nitroglycerin in Metastatic Non-Squamous-Non–Small Cell Lung Cancer, NCT01171170). After the patient's disease had remained stable and her clinical condition had remained good for 12 months, new subcutaneous lesions developed on her scalp and thorax. A surgical biopsy of one of the scalp lesions was performed, and the pathologist diagnosed a combined large cell and small cell neuroendocrine carcinoma (Table 4.1, report IVa; Figures. 4.1F–1J). Carboplatin-etoposide chemotherapy was subsequently initiated; however, the scalp metastases showed no response.

After discussion in a multidisciplinary team, all the biopsy findings were revised and the tumor was reclassified as an atypical carcinoid with a mitotic index of 6/10 (HPFs). This reclassification was supported by the results of an additional KI-67 stain (15%–20%) (Table 4.1, report IVb). Peptide receptor radionuclide therapy with octreotide was administered, but because of side effects, it was halted at the patient's request. Eventually, the patient was referred for participation in a randomized phase 2 trial for atypical carcinoid (Three- Arm Trial to Evaluate Pasireotide LAR/Everolimus Alone/in Combination in Patients with Lung/Thymus NET [NCT01563354]) and was assigned to treatment with the mammalian target of rapamycin inhibitor everolimus. Currently, the

patient is in a good clinical condition and her disease is stable 14 months after initiation of everolimus and 42 months after the initial diagnosis.

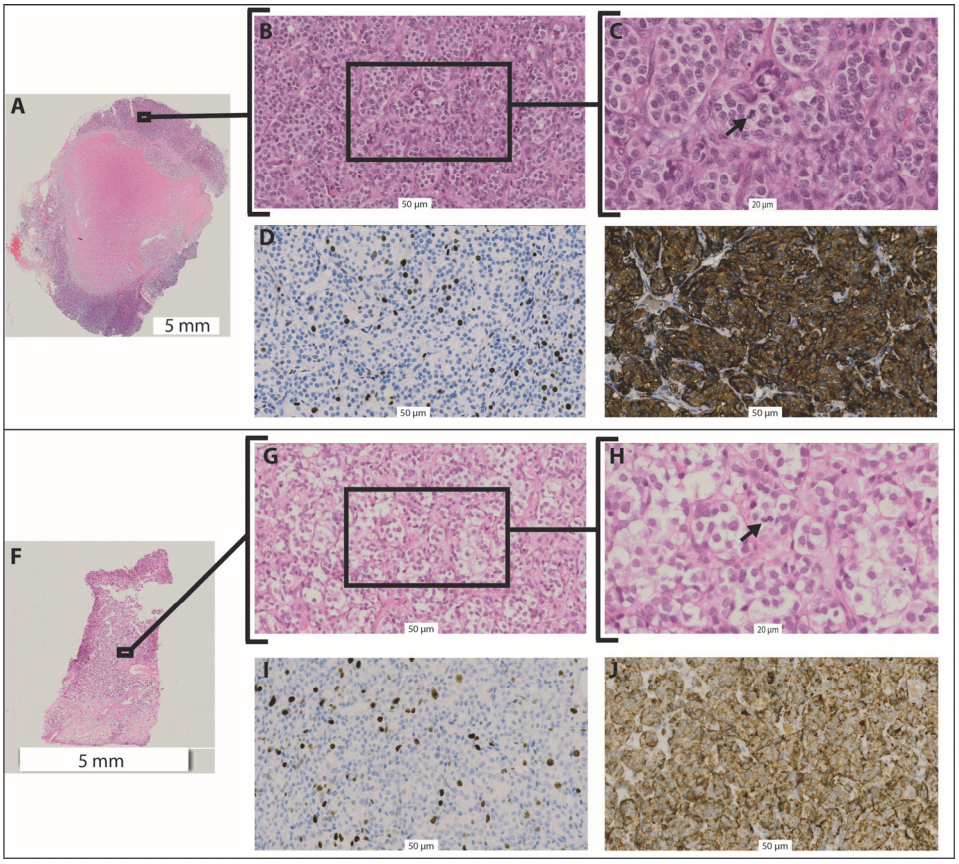

Figure 4.1 Pathological slide overview of the subcutaneous posterior thoracic wall lesion and scalp lesion from the same patient that was, after multiple revisions, classified as atypical carcinoid.

Posterior thoracic wall biopsy

A Overview slide, tumor with central necrosis.
B 20 fold magnification: shows an organoid growth pattern with nests of large cells with abundant cytoplasm and monotonous nuclei. There is no dot-like necrosis.
C 40 fold magnification: in the center a single mitosis (arrow head).
D/E Positive immunohistochemical staining for Ki-67 and Chromogranin-A

Subcutaneous scalp biopsy

F Overview slide, slight crush artefacts are observed in the left top in a possibly less well preserved biopsy.
G 20 fold magnification: slightly more diffuse pattern with occasional nests, showing large cells with abundant clear cytoplasm, nucleoli. There is no presence of necrosis.
H 40 fold magnification: in the center a single mitosis (arrow head).
I/J Positive immunohistochemical staining for Ki-67 and Chromogranin-A

Table 4.1 Overview of pathological sampling methods, recorded diagnosis and timespan

	Recorded information from pathology reports					
Report	Time	Diagnosis	Location	Mitosis*	IHC staining pattern	Mutation
I	0 days	Poorly differentiated carcinoma	Excision Subcutaneous (posterior thoracic wall)	No index	MNF+, CK7-, CK20-, P63-, CD45- S100-, HMB45-	-
II	8 days	NSCLC; immunohistochemical pattern LCNEC	Fine needle aspiration breast	19	TTF1+, Chromogranin A+, Synaptophysin+, MelanA-, Estrogen-, GCDFP-, CK7-, WT1-, Calcitonin-	KRAS WT, EGFR WT, ALK-
II *(revision of report I)*	8 days	NSCLC; immunohistochemical pattern LCNEC	Excision Subcutaneous (posterior thoracic wall)	No index[i]	See report I	-
Iva	12 months	NEC: partly small cell partly round cell (variant SCLC)	Surgical biopsy Subcutaneous (scalp)	No index	TTF1+, Chromogranin A+, Synaptophysin+, CD56+, p40-, CK7-, CK5/6-, Napsin A-, Calcitonin-	-
IVb *(revision of report IVa)*	13 months	Atypical carcinoid	Surgical biopsy Subcutaneous (scalp)	6	Ki-67 15-20%†	-

* Assessed by calculation of 10 high power fields (2 mm^2); † Assessed at final revision after multidisciplinary lung meeting; [i] 8 mitosis in a single HPF field

Abbreviations: NSCLC, non-small cell lung cancer; LCNEC, large cell neuroendocrine carcinoma; NEC, neuroendocrine carcinoma; SCLC, small cell lung carcinoma; WT, wild-type; IHC, immunohistochemical

Because of the unusual clinical development of this case and the difficulty in reaching a histological diagnosis, we requested that three expert pathologists perform a blind revision of the two histological specimens. They were unaware of the fact that the specimens belonged to one and the same patient and did not know the location of the primary tumor. Two pathologists (B and C) diagnosed atypical carcinoid/neuroendocrine tumor grade 1–2 in both the initial biopsy sample and the biopsy sample of the metastasis on the scalp (Table 4.2). Pathologist A preferred LCNEC on the basis of the mitotic count but stressed that the findings in this case bordered on meeting the criteria for atypical carcinoid (i.e., 11 mitosis per 10 HPFs); in the second biopsy sample pathologist A diagnosed an atypical carcinoid.

Table 4.2 Overview of revision diagnoses established by blinded revision by three expert pathologists

	Subcutaneous posterior thoracic wall lesion		*Subcutaneous scalp lesion*	
Pathologist	Diagnosis	Mitosis* /necrosis	Diagnosis	Mitosis* /necrosis
A	Pulmonary origin: strictly speaking LCNEC (close to atypical carcinoid) Extra pulmonary: NET grade 2	11 mitosis KI-67: 10% Punctate necrosis	Pulmonary origin: atypical carcinoid Extra pulmonary: NET grade 2	3 mitosis KI-67: 10% Punctate necrosis
B	Atypical carcinoid	5-6 mitosis difficult due to apoptotic cells KI-67: - No necrosis	Atypical carcinoid	5-6 mitosis difficult due to apoptotic cells KI-67: - No necrosis
C	NET grade 1-2	<10 mitosis KI-67: - No necrosis	NET grade 1-2 / carcinoid	<10 mitosis KI-67: - No necrosis

* Assessed by calculation of 10 high power fields (2 mm^2)
Abbreviations: NET, neuroendocrine tumor; LCNEC, large cell neuroendocrine carcinoma; HPF, high power field

Discussion

In the 2015 World Health Organization classification, PNETs form a cluster of diseases that can be subdivided into prognostic subtypes with possible implications for choice of therapy. Typical carcinoids are recognized by two or fewer mitoses per 2 mm^2 and absence of necrosis, atypical carcinoids have more than two but no more than 10 mitoses and/or occasional punctate necrosis, and LCNEC and small cell carcinoma (SCLC) have more than10 mitoses per 2 mm^2 and/or central necrosis. Moreover, LCNEC can be distinguished from SCLC on the basis of observation of abundant cytoplasm, presence of nucleoli, or large cell size[1].

Availability of only small biopsy samples and sporadic exposure may limit the diagnostic accuracy of the current pNET classification. Also, true mitoses may be difficult to distinguish from pycnotic nuclei, which would explain part of the interobserver variation in assessing the mitotic index[2,3]. Addition of prognostic markers to the current WHO classification may aid the pathologist in difficult cases. Here, the low Ki-67 staining index could have guided the diagnoses toward a low-grade tumor at initial assessment because a low Ki-67 index is not consistent with aggressive pNET behavior[4]. Nevertheless, the value of the Ki-67 index and its routine use in pNET are still under debate[5], although it might be of use in (crushed) biopsy specimens, in which the mitotic index is frequently discordantly lower than the Ki-67 index[6].

After receiving the different pathological diagnoses and observing the disease progression in this case, we administered various treatments. Treatment of LCNEC is still debated and favors either a non–small cell lung carcinoma- or SCLC-based chemotherapy regimen; however, some experts prefer the latter[7]. In somatostatin receptor–positive pulmonary carcinoids, peptide receptor radionuclide therapy showed a morphological and clinical response in 28% and 38% of patients[8]. Additionally, everolimus combined with the somatostatin analogue lanreotide showed a trend toward providing longer progression-free survival than did placebo and lanreotide in a subgroup of functional (secretory) pulmonary carcinoids from the Everolimus Plus Octreotide Long-Acting Repeatable for the Treatment of Advanced Neuroendocrine Tumours Associated with Carcinoid Syndrome study[9].

As shown in this case study, classification of neuroendocrine tumors with an intermediate grade may be difficult and may lead to different diagnoses over time with different therapeutic treatment implications. Ultimately, clinical decision making in a multidisciplinary team was essential for the management of this patient's disease. In our view and that of the European Neuroendocrine Tumor Society (ENETS), multidisciplinary meetings and consultation of an expert center for pathological and clinical assessment are essential for optimal treatment in patients with pNETs[10]. Also clinicians should be aware of the difficulties pathologists can have in diagnosing pNETs.

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Chapter 5

A population based analysis on application of WHO-nomenclature in pathology reports of pulmonary neuroendocrine tumors

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Abstract

Pulmonary neuroendocrine tumors (pNETs) are difficult to classify. We performed a population-based analysis to investigate the pNETs nomenclature application in daily pathology practice

Conclusions from pathology reports (2003-2012) describing carcinoids, (large cell) neuroendocrine carcinomas ((LC)NEC) and carcinomas with neuroendocrine features/differentiation were retrieved from PALGA (the Dutch Pathology Registry) by queries on location and diagnosis and screened for terminology. Cases with non-pulmonary/unknown origin and small cell lung cancer (SCLC) were excluded. Diagnoses were clustered into subgroups and retrieved terminology was compared to WHO 2015 diagnoses. By means of an online questionnaire, interpretation of the retrieved non-WHO nomenclature from pathology reports was evaluated (physicians (N=35) / pathologists (N=19).

3216 unique pathology conclusions with 55 different pNET diagnoses (*N*=3052) and 20 uncertain diagnoses (*N*=164) were analyzed. Non-WHO nomenclature was used in 15% (*N*=488) of diagnoses. Diagnoses could be clustered into carcinoids (*N*=1086), NEC (*N*=1316), carcinomas with neuroendocrine features/differentiation (*N*=624) and unspecified pNETs (*N*=26). Non-WHO nomenclature within these clusters was found for 7% of carcinoids, 20% of NECs, 13% of carcinomas with neuroendocrine features/differentiation and 100% of unspecified pNETs and was observed more often in conclusions of biopsy/cytology specimens (62/12%) compared to resection specimens (26%). Questionnaire analysis revealed that 4/19 non-WHO nomenclature diagnoses were uniformly interpreted (>50% agreement) by physicians and 10/19 diagnoses by pathologists.

In 15% of pNETs other than SCLC, a non-WHO nomenclature diagnosis was provided, more frequently on smaller specimens. The interpretation was different between physicians and pathologists. Application of uniform nomenclature among all clinicians is advocated.

Introduction

Pulmonary carcinoids and large cell neuroendocrine carcinomas (LCNEC) represent distinct subcategories of lung cancer, which together with small cell lung cancer (SCLC) are referred to as pulmonary neuroendocrine tumors (pNETs). Pulmonary carcinoids and LCNEC are orphan disease due to the low incidence whereas SCLC is encountered more frequently[1]. Initially the neuroendocrine tumor was recognized and described as a "Karzinoid" of the abdomen by Siegfried Oberndorfer[2], since then, the diagnosis of pNETs has evolved into the WHO classification of 2015[3]. This classification is currently also recommended and endorsed by the European Neuroendocrine Tumor Society (ENETS) for use in daily pathology practice to keep classification among pathologist consistent and comparable[4].

Over the past decades the classification of pNETs has changed several times and this has led to inconsistent application of nomenclature. Since the WHO classification of 1981, the classification of SCLC has been simplified from oat-cell, intermediate-cell and combined-cell type to either SCLC or combined SCLC. The entity LCNEC was introduced by the WHO in 1999 after defining a pNET that lay in between the spectrum of the atypical carcinoid and SCLC[5]. Alternative classification schemes proposed by Gould (1983)[6], Capella (1995)[7] and Huang (2002)[8] focused on morphological differentiation (well, intermediate and poorly differentiated neuroendocrine tumors/carcinomas), similar to the present classification of gastro-pancreatic NET (grade I-III) (Rindi, 2007)[9,10].

To keep classification consistent, the recently proposed alternative classification for pNETs (i.e. mitosis, necrosis and Ki67 based[11]) has not been adopted in the 2015 WHO scheme. Therefore, the 2015 classification of pNETs matches that of 2004 and 1999, with the exception of transferring LCNEC from the large cell carcinoma category to the pNETs category. However, implementation of the pNETs classification in daily practice can be difficult because of several factors, including, 1) infrequent exposure in general pathology practice, 2) recognized inter-observer variation, even among experienced pathologists[12-14], 3) the requirement of surgical resected tissue for assessment of all morphological criteria, rendering diagnosis on small biopsies and cytology tenuous, 4) the difference between the pulmonary, thyroid and gastro-intestinal classification system of NET with regard to advised nomenclature and mitosis/Ki67 evaluation; and finally, 5) the fact that classification implies categorization, for which criteria are used, aiming to separate into classes with prognostic relevance (e.g. typical and atypical carcinoid), while the underlying tumor biology might not be so easily separated. All these factors may cause variation in use of nomenclature for classification of pNETs.

In this study we investigated whether the nomenclature and interpretation applied in the routine pathology practice reports to diagnose typical/atypical carcinoids, LCNEC and carcinomas/non-small cell lung cancer (NSCLC) with neuroendocrine immuno-histochemical (IHC) staining conformed to that advised by the WHO 2015 classification on a population basis, whilst taking into account whether the diagnosis was made on surgically/non-surgically obtained specimens or cytology. To evaluate the clinical significance of non WHO-nomenclature, physicians and pathologists were invited to re-assign cases to the established WHO diagnosis (2015).

Materials and methods

PALGA, the Dutch pathology registry

All data for this study were retrospectively retrieved from PALGA[15] (the nationwide network and registry of histo- and cytopathology in the Netherlands). PALGA automatically archives conclusions from pathology reports composed by the 55 different Dutch pathology departments since 1971 (with complete report coverage from 1991). Pathologists attach restricted uniform codes to pathology reports with information on tumor localization, sampling method (i.e. biopsy, excision, resection or cytology) and the pathological diagnosis. Codes are transcribed into SNOMED (Systematized Nomenclature of Medicine) codes, attached to a patient pseudonym and registered by PALGA. The SNOMED codes can be found in the online PALGA thesaurus[16].

Retrieval of written diagnostic conclusions of pathology reports

Written conclusions (diagnoses) of pathology reports describing carcinoids, LCNEC, neuroendocrine carcinomas and large cell carcinoma (LCC)/NSCLC/carcinomas with neuroendocrine differentiation diagnosed from 01-01-2003 to 31-12-2012 were retrieved. This retrieval process contained four complementary database queries (Table 5.1) including SNOMED codes on anatomical location and diagnosis (search 1), in combination with free text keywords (e.g. carcinoma + endocrine and lung, search 2-4). Keywords were chosen to increase sensitivity as conclusions may sometimes lack specific SNOMED coding. Additionally, wildcards were included in both the SNOMED code and keyword queries to detect variation in spelling.

Table 5.1 Selection of conclusions of pathology reports from PALGA: depicted by search order and complementary codes

Search order	SNOMED				Keywords
	Sampling location	Code	Histology selection	Codes*	Free text search
Search (1)	Lung	T28___	(metastasis) NET grade I	M8242_	none
N=3911	Bronchus	T26___	(metastasis) NET	M8244_	
	Pleural effusion	T2Y6__	(metastasis) NET II	M8246_	
	Pleural cavity	T2Y7__	(metastasis) LCNEC	M8247_	
	Pleurae	T29___	(metastasis) Atypical carcinoid	M8241(3/6)	
	Mediastinum	TY23__	(metastasis) Carcinoid	M8240(3/6)	
Search (2)	Lung	T28___	(metastasis) NSCLC	M8047_	(Endocrien OR Endocrine)
N=3254	Bronchus	T26___	(metastasis) LCC	M8012_	
	Pleural effusion	T2Y6__	(metastasis) Carcinoma	M8010(3/6)	
	Pleural cavity	T2Y7__	(metastasis) SqCC	M807_(3/6)	
	Pleurae	T29___	(metastasis) AdC	M814_(3/6)	
	Mediastinum	TY23__	(metastasis) SCLC	M8141_(3/6)	
Search (3)	none		(metastasis) NSCLC	M8047_	(Endocrien OR Endocrine)
N=442			(metastasis) LCC	M8012_	AND Lung
			(metastasis) Carcinoma	M8010(3/6)	
Search (4)	none		(metastasis) NET grade I	M8242_	Lung
N=382			(metastasis) NET	M8244_	
			(metastasis) NET II	M8246_	
			(metastasis) LCNEC	M8247_	
			(metastasis) Atypical carcinoid	M8241(3/6)	
			(metastasis) Carcinoid	M8240(3/6)	

** codes referred to here are the SNOMED (Systemized Nomenclature of Medicine Clinical Terms) codes*

Abbreviations: PALGA, the Dutch Pathology Registry; NET, neuroendocrine tumor; LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung cancer; LCC, large cell carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma; SCLC, small-cell lung cancer

Screening process of pathology diagnosis

Selected conclusions were clustered according to patient pseudonyms (i.e. in case of multiple reports). Screening was carried out by one reader (JLD) and for advice in difficult cases RJS was consulted. The following data were extracted: tumor sampling location (e.g. lung, liver or lymph node), diagnosis recorded in the conclusion, and origin of primary tumor in case of a metastasis. Also the sampling method was extracted and subdivided into non-surgically obtained biopsies (trans/endo bronchial and needle), cytology specimens (bronchiolar lavage, brush and fine needle) and resection specimen (any surgical resection). Subsequently several evaluation steps were performed. First, in cases were a clinical revision was performed (i.e. a second opinion), the revision diagnosis was recorded. Second, if a preferred diagnosis was mentioned, only that diagnosis was recorded. Third, if an uncertain (i.e. differential) diagnosis without a preference diagnosis was reported, all debated diagnoses were recorded. Fourth, diagnoses established on an intra-thoracic lymph node, pleural effusion or the mediastinum were recorded as metastasis of pulmonary origin whenever the origin was confirmed in the conclusion text/SNOMED code. Fifth, a tumor sampled from a location other than the pulmonary tract and the conclusion/SNOMED code mentioned lung as primary origin, or immunohistochemical pattern was lung-specific, this diagnosis was recorded as lung origin. Finally, if multiple conclusions were available for a single patient (e.g. duplicate or corresponding conclusions on single tumor), the diagnosis was established on the most extensive tissue sampling method (surgically resected tissue>non-surgically obtained biopsy>cytology) or from the results from the final revision.

Selection of conclusions of pathology reports

Report conclusions were excluded from analysis when origin of the primary tumor was non-lung or of undefined origin (including undefined cases of mediastinal tumors and intrathoracic lymph node metastasis), when the diagnosis was non-neuroendocrine tumor (differentiation/features) or confirmed SCLC disease and whenever the diagnosis was established on an autopsy specimen.

Data analysis

All diagnoses were compared to the 2015 WHO manual and clustered into either non-WHO nomenclature, or classified according to the WHO nomenclature. The 2015 WHO pNET classification was chosen because the diagnostic criteria and advised nomenclature has been practically unchanged compared to the 1999 and 2004 classification. The diagnosis LCC/NSCC/carcinoma with neuroendocrine features was added to the analysis

as this diagnosis is not described in the WHO 2015, however was frequently retrieved on screening of conclusions. Subsequently, all diagnoses were clustered into sub-groups by applying the criteria listed in Table 5.2 (carcinoid, high-grade neuroendocrine carcinoma, LCC/NSCLC/carcinoma with neuroendocrine features/differentiation, neuroendocrine tumor not otherwise specified (NOS), or unclear diagnosis).

Table 5.2 Words used to cluster diagnosis retrieved after screening of conclusions

Clustered diagnosis	Words required for clustering *Word (1)*	*And / Or*	*Word (2)*
Carcinoids	Carcinoid Neuroendocrine tumor		Low-grade Well differentiated Highly differentiated Grade I-II Intermediate differentiated
High-grade neuroendocrine ca.	Neuroendocrine (large/non-small cell) ca. Combined large/small cell ca.		High-grade Poorly differentiated Intermediate cell type
Ca. with neuroendocrine features/differentiation (large/non-small cell)	Ca. (large/non-small cell) AdC SqCC		(IHC) Neuroendocrine features (IHC) Neuroendocrine differentiation Endocrine features
Neuroendocrine tumors	Unclear if either ca. or carcinoid		
Uncertain diagnosis	All conclusions with uncertainty (i.e. more than 2 possible differential diagnoses)		

Abbreviations: IHC, immunohistochemical; Ca, carcinoma; AdC, Adenocarcinoma; SqCC, squamous cell carcinoma.

Questionnaire analysis

An online questionnaire (SurveyMonkey, Inc, Palo Alto, CA was constructed and completed by 19 (expert) pathologists and 35 physicians. In this questionnaire, all retrieved non-WHO nomenclature diagnoses with a frequency of ≥5, were listed and participants were asked how they would interpret the diagnosis. Participants' responses were limited to one of the following five WHO (2004/2015) categories: typical carcinoid, atypical carcinoid, carcinoid (NOS), (combined) LCNEC and (combined) SCLC. Moreover, NSCLC with immunohistochemical neuroendocrine differentiation on IHC was added. Finally, a "don't know" cluster was added for cases where the previous categories did not fit according to the questionnaire participants.

Statistical analysis

The χ^2 test was used to compare categorical variables. All *p*-values were two sided and *P*-values <0.05 were considered statistically significant. All analyses were performed using SPSS (version 22.0 for Windows (SPSS Inc. Chicago. IL)).

Results

Selection of pathology report conclusions and clustering of subgroups

Queries run by PALGA retrieved 7989 pathology report conclusions (Table 5.1). After screening and applying the proposed exclusion criteria, conclusions of 3216 unique patient cases were included for nomenclature analysis (flowchart Figure 5.1). Seventy five unique diagnoses could be retrieved, including 55 different pNET diagnoses (retrieved from 3052 conclusions) and 20 unclear (differential) diagnoses (retrieved from 164 pathology conclusions), further described in Table S5.1. Clustering of the pNET diagnoses was performed and characteristics are presented in Table 5.3. The majority of diagnoses were established on lung tissue and the most frequently used sampling method was surgical resection, closely followed by the non-surgical biopsies (Table 5.3).

Application of the WHO nomenclature

Non-WHO nomenclature was used in 488 out of 3052 (15%) cases with a pNET conclusion. The retrieved conclusions with non-WHO nomenclature could be separated into 24 different diagnoses (Table S5.1). In the cluster of carcinoid diagnoses 7% (78 cases) had non-WHO nomenclature and this percentage did not change significantly over time (Figure 5.2 and Table 5.3). Besides non-WHO nomenclature, 35% of diagnoses in the carcinoid cluster did not discriminate between typical and atypical carcinoid but were diagnosed as carcinoid NOS (376 cases, Table S5.1). Moreover, the diagnosis was established on a resection specimen in 28% of the carcinoid NOS cases (220 cases), although this decreased over time (35% (118 cases) ≤2007 vs. 23% (102 cases) ≥2008 (P<0.001)). In the cluster high-grade neuroendocrine carcinomas 20% of diagnoses (262 cases) were classified as non-WHO nomenclature and this percentage decreased significantly over time (Figure 5.2 and Table 5.3). Finally, in the LCC/NSCLC/carcinoma with neuroendocrine features/differentiation cluster, 13% of diagnoses (82 cases) provided non-WHO nomenclature, which did not change over time. Overall, non-WHO nomenclature diagnoses were established more often on non-surgically obtained biopsies and cytology specimens than on resection specimens (Table 5.3 and Figure 5.3a and 5.3b)

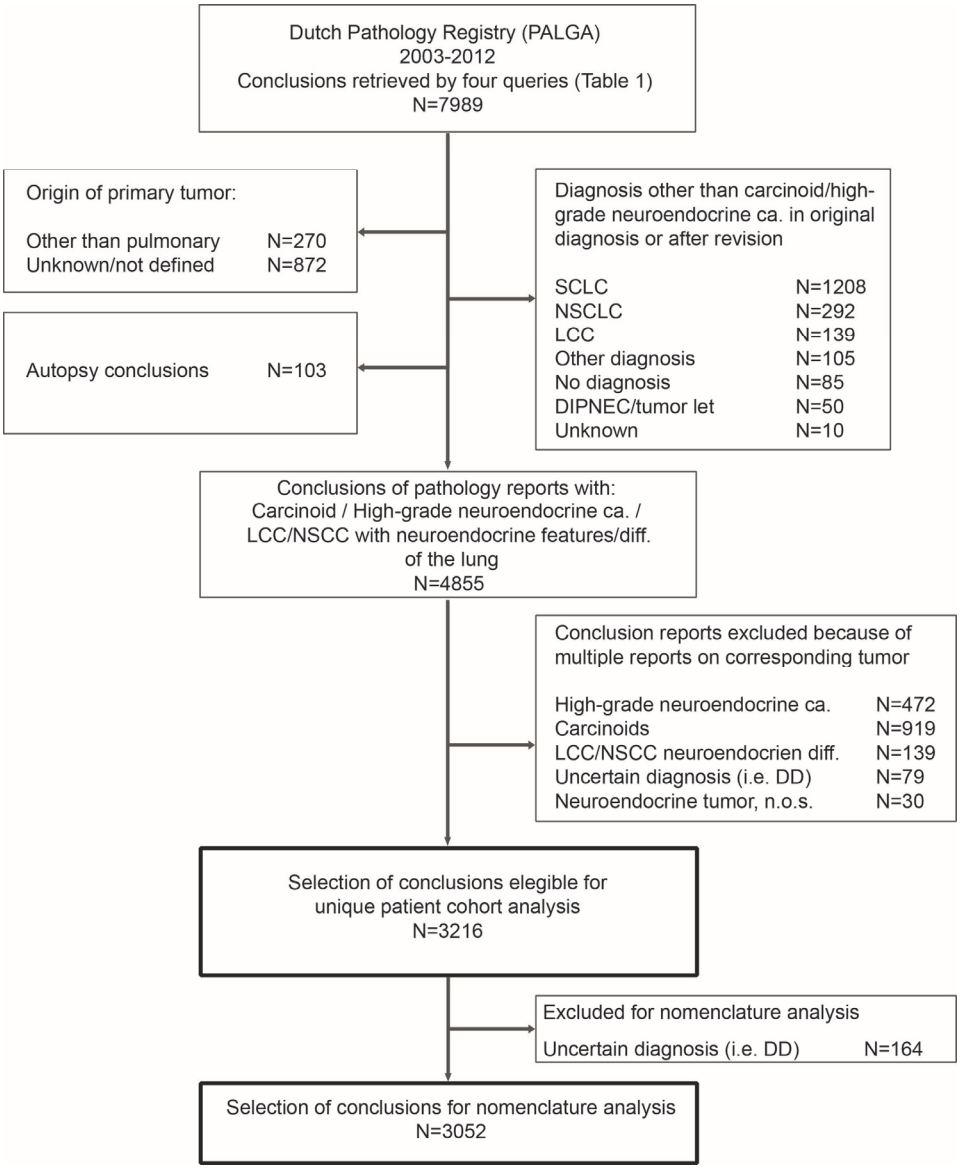

Figure 5.1 Flowchart overview of selection of conclusions from pathology reports after manual screening of 7989 pathology conclusion texts. After application of exclusion criteria, 3052 unique patient conclusions were eligible for diagnostic nomenclature analysis
Abbreviations: PALGA, the Dutch Pathology Registry; SCLC, small cell lung cancer; NSCC, non-small cell cancer; LCC, large cell carcinoma; DIPNEC, diffuse idiopathic pulmonary neuroendocrine cell hyperplasia; NEC, neuroendocrine carcinoma; NOS, not otherwise specified; DD, differential diagnosis; Ca, carcinoma; Diff, differentiation.

Table 5.3 Characteristics of written conclusions of pathology reports

Variable	Total cohort			Non-WHO nomenclature cohort			
		Time period			Time period		
	Total	≤2007	≥2008	*Total*	≤2007	≥2008	vs.
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	*p**
Conclusions	3052(100)	1280 (42)	1772 (58)	448 (15)	205 (46)	243 (54)	0.076
Sampling location							
Lung	2297 (75)	890 (43)	1161 (57)	246 (55)	127 (52)	119 (48)	-
Metastasis (lymph node or distant)	755 (25)	185 (34)	368 (24)	202 (45)	78 (39)	124 (61)	-
Sampling method							
Surgically resected	1517 (50)	616 (44)	785 (56)	116 (26)	53 (46)	63 (54)	-
Non-surgical obtained biopsy**	1355 (44)	417 (39)	660 (61)	278 (62)	127 (46)	151 (54)	-
Cytology***	180 (6)	42 (33)	84 (67)	54 (12)	25 (46)	29 (54)	-
Diagnosis cluster							
Carcinoid	1086 (36)	480 (44)	606 (56)	78 (7)	50 (8)	28 (6)	0.125
High-grade neuroendocrine ca.	1316 (43)	503 (38)	813 (62)	262 (20)	121 (24)	141 (17)	0.003
(LCC/NSCC) ca. neuroendocrine diff./features	624 (20)	282 (45)	342 (55)	82 (13)	41 (15)	41 (12)	0.348
Neuroendocrine tumor NOS	26 (1)	15 (58)	11 (42)	26 (100)	15 (58)	11 (42)	-

* Chi-square used to test if non-WHO nomenclature increased/decreased significantly between time periods ≤2007 and ≥2008 (i.e. non WHO-nomenclature vs. WHO nomenclature)

** Here the non-surgically obtained biopsy is a referral for the cluster of trans/endo bronchial biopsy specimens and needle biopsy specimens

*** Fine needle aspiration (no histology), brush and bronchial lavage specimens

Abbreviations: vs., versus; NSCC, non-small cell cancer; LCC, large cell carcinoma; ca, carcinoma; NOS, not otherwise specified

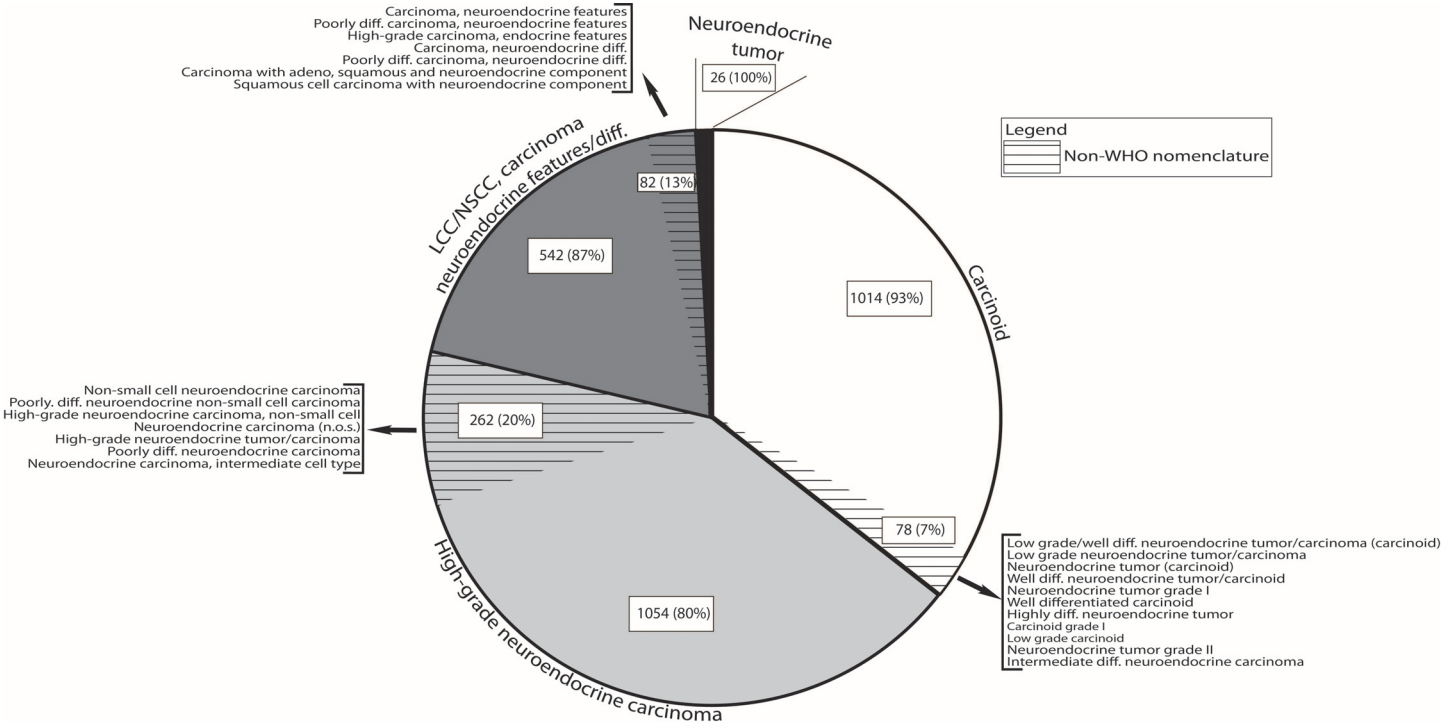

Figure 5.2 An overview is given of diagnoses with non-WHO nomenclature versus those diagnosed according to the WHO nomenclature, and separated for the following clusters: carcinoid, high-grade neuroendocrine carcinoma and LCC/NSCC, carcinoma with neuroendocrine features or differentiation. The exact number of all retrieved non-WHO nomenclature diagnoses can be found in Table S5.1.
Abbreviations: WHO, World Health Organization; NOS, not otherwise specified; Diff, differentiation; NSCC, non-small cell carcinoma; LCC, large cell carcinoma.

Diagnosis on biopsy and cytology specimens

Carcinoids were generally diagnosed on surgically resected tissue specimens (779 cases, 72%). Nonetheless, 19% (88 cases) and 26% (63 cases) of typical and atypical carcinoid diagnoses were established on non-surgically obtained biopsies, respectively (Figures 5.3a and 5.3c). The diagnosis LCNEC was established on non-surgically resected biopsies in 56% (546 cases), and a strong increase was observed in diagnosis on non-surgically obtained biopsies and cytology over time (≤2007 120 cases (43%) vs. ≥2008 326 cases (62%), *P*<0.001) (Figure 5.3d).

Clinical interpretation of non-WHO diagnostic terminology

With use of an online questionnaire, 35 physicians and 19 pathologists interpreted non-WHO terminology. In only 1 out of 7 non-WHO nomenclature diagnoses that clustered as carcinoid, uniformity in interpretation among physicians exceeded 50% (table S5.2A). The non-WHO diagnoses “Neuroendocrine tumor grade I” and “grade II” had over 50% of agreement among pathologists, whereas these diagnoses were unclear for physicians. In the high-grade neuroendocrine carcinoma cluster, 1 out of 7 and 4 out of 7 non-WHO diagnoses had ≥50% agreement among physicians and pathologists, respectively. These diagnoses included “NSCLC neuroendocrine carcinoma”, “poorly differentiated neuroendocrine NSCLC”, “high-grade neuroendocrine carcinoma NSCLC”, and “high-grade neuroendocrine tumor/carcinoma” (table S5.2B). Among physicians, combined LCNEC was frequently interpreted as NSCLC with neuroendocrine IHC differentiation. Finally, in the cluster of LCC/NSCLC/carcinomas with neuroendocrine features/differentiation, 2 out of 4 and 3 out of 4 non-WHO diagnoses scored higher than 50% agreement among physicians and pathologists, respectively (Table S5.2C). Remarkably, all diagnoses of LCC with either neuroendocrine features or differentiation were often interpreted as (combined) LCNEC by physicians. However, interpretation of diagnosis with neuroendocrine features/differentiation improved substantially towards NSCLC with neuroendocrine IHC differentiation when either the term IHC staining or a certain morphological differentiation was added (i.e. squamous carcinoma (SqCC)/ adenocarcinoma (AdC)) (Table S5.2C).

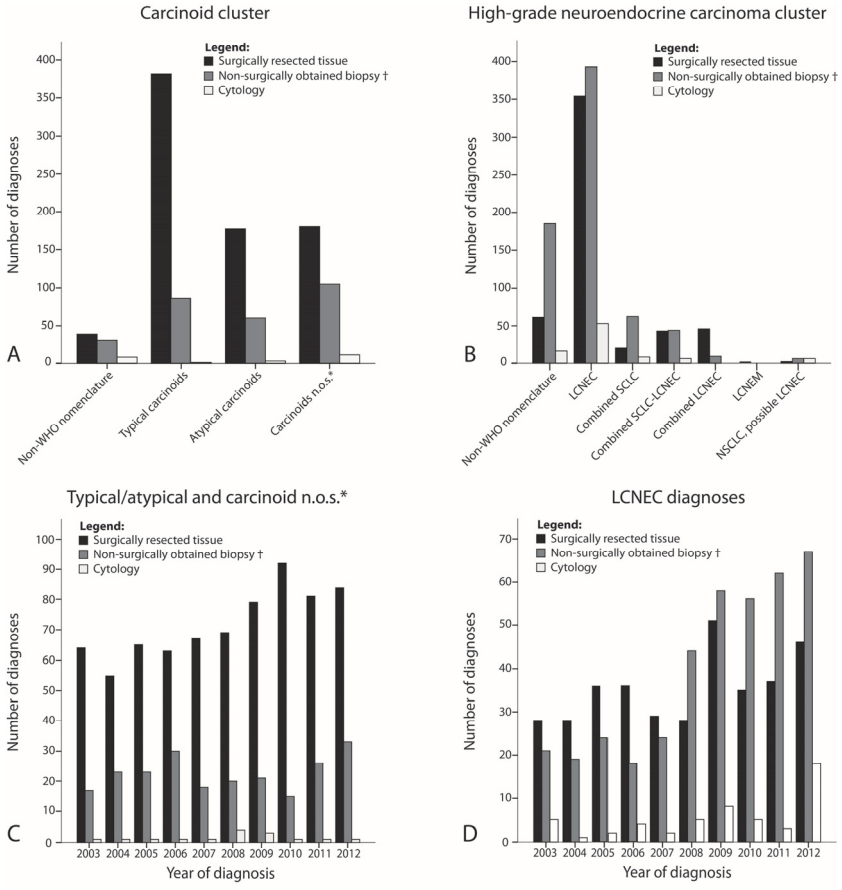

Figure 5.3 **A**) Overview of all diagnoses clustered as carcinoid and separated for type of sampling method. **B**) Overview of all diagnoses clustered as high-grade neuroendocrine carcinomas and separated for type of sampling method. **C**) Overview of sampling methods used to diagnose all typical/atypical and carcinoid NOS diagnoses from 2003 to 2012. **D**) Overview of sampling methods used to diagnose LCNEC from 2003 to 2012.

* The carcinoid NOS diagnosis presented here is a combination of the diagnoses "carcinoid, no atypical features; carcinoid, unsure if typical/atypical, and carcinoid NOS from Table S5.1"

† Here the non-surgically obtained biopsy is a referral for the cluster of trans/endo bronchial biopsy specimens and needle biopsy specimens.

Abbreviations: WHO, World Health Organization; NOS, not otherwise specified; SCLC, small cell lung cancer; LCNEC large cell neuroendocrine carcinoma; NSCLC, non-small cell lung cancer; LCNEM, large cell carcinoma with neuroendocrine morphology but not neuroendocrine immunohistochemistry.

Discussion

Uniformity in diagnosis and treatment is essential to increase the quality of care for patients. In this population-based analysis, we showed that in routine pathology practice the nomenclature of pNETs regularly deviates from that advised by the WHO-classification and that non-WHO nomenclature containing diagnoses are confusing for physicians. Moreover, non-surgically obtained biopsies are often rendered for pNET diagnostic purpose, even though current WHO classification criteria for diagnoses on biopsies are deemed insufficient.

In all likelihood, physicians treating patients with a pNET are insufficiently aware of the difficulties pathologists encounter when diagnosing these tumors. Also, pathologists might be unaware of the problems physicians have when confronted with a diagnosis that deviates from established nomenclature. Although our questionnaire had a limited amount of participants, clinical relevant difference in interpretation of non-WHO diagnoses between pathologists and oncologists was observed. It might be that historical or non-pulmonary classification terminology can be ambiguous for physicians, but straightforward for pathologists. To prevent differences in interpretation among physicians and pathologists, increased awareness of this issue is needed.

Several explanations may account for application of non-WHO nomenclature in pathology report conclusions. First, a categorical classification system such as the WHO, sometimes arbitrarily, aims to segregate prognostic classes. Consequently cases may be scored as borderline between distinct classes, whereas nature may be more flexible. The difficulties experienced by pathologists to categorize such cases, may lead to the use of non-WHO-terminology as a best approximation. Additionally, infrequent exposure in daily practice of (non-expert) pathologists to pulmonary carcinoids and LCNEC cases may lead to an unintentional application of incorrect nomenclature. Also, as pathologists diagnose both gastrointestinal as well as pulmonary NETs and nomenclature is different for these classifications, application of nomenclature may be confusing in daily practice[17]. Finally, we observed that non-WHO nomenclature was most often used in diagnoses established on non-surgically obtained biopsy specimens. This is not surprising as a relevant diagnostic category was lacking in the WHO 2004 classification[18], and uncertainty probably is an important factor driving non-WHO nomenclature usage

Besides establishment of non-WHO nomenclature diagnosis, there was a significant proportion of carcinoid NOS diagnosis in the retrieved reports. Such a diagnosis is incomplete, as sub-classification into typical and atypical carcinoid is essential for

estimation of prognosis[19]. Moreover, it has been shown that the carcinoid NOS diagnosis established in daily practice can be reclassified into typical or atypical carcinoid[20]. Possibly, description of the mitosis/necrosis in the pathology report, if available, could have guided the physician in carcinoid NOS cases. However, the majority of patients with carcinoid disease are not diagnosed in a specialist center, nor visit one during follow-up of disease[21]. Therefore, the primary pathology report conclusion is what most physicians will base their subsequent decisions and treatment on and for this reason an as adequate as possible classification/conclusion is required.
We found that the diagnosis NSCLC with neuroendocrine differentiation (IHC) or features was regularly used in daily pathology practice. The value of NSCLC with neuroendocrine differentiation (IHC) but without neuroendocrine morphology is still under debate, as no clear relation with prognosis has been demonstrated[22-28]. Therefore, the recently revised WHO manual proposes not to stain for neuroendocrine IHC markers in NSCLC lacking neuroendocrine morphology[3]. As shown in our questionnaire inclusion of neuroendocrine IHC marker results in cases were no neuroendocrine morphology is observed, can confuse physicians. Nevertheless, if pathologists do add IHC results, the diagnostic nomenclature preferably will include the exact description of neuroendocrine differentiation features (morphology and IHC). And thus avoids ambiguous nomenclature such as “NSCLC with neuroendocrine features or “LCC with neuroendocrine differentiation”.

Several steps can be taken to reduce non-WHO nomenclature usage and improve consistency in diagnostic terminology used in pathology reports. One way would be to always explicitly state the classification system (i.e. gastrointestinal or thoracic), as proposed by gastrointestinal NET experts during a consensus meeting[29]. Another approach would be to implement structured and uniform pathology reporting, so that pathologists are forced to adhere to the WHO-nomenclature, a process that currently is being implemented in the Netherlands[30]. Finally, central reviewing of equivocal cases could be advised; this might reduce interobserver variation and non-WHO nomenclature diagnoses. However, central review is not practically feasible for all established pNET diagnoses and does not guarantee improvement of prognostic classification in pulmonary carcinoids[14]. In a recent guideline, the ENETS advised that problematic carcinoid cases may well benefit from expert pathologists revision and all cases need to be discussed in a multidisciplinary meeting at all times[4].

Besides incorrect or incomplete nomenclature usage, we observed that pulmonary carcinoids and LCNEC were often diagnosed on non-surgically obtained biopsy specimens despite the lack of clear histological biopsy criteria in the WHO 2004

classification. In the new WHO 2015 classification[3], this is unresolved in carcinoids as clear criteria for biopsy specimens remain lacking; mainly because mitoses cannot reliably be assessed here. In high grade neuroendocrine carcinomas, suspicion of LCNEC on a biopsy specimen should now be referred to as "NSCLC, possible LCNEC" although explicit diagnostic criteria are also not provided[31], but have been suggested[32]. Our findings stress the need for revision of the specimen, especially pNETs on biopsy specimens aiming to clarify the underlying histology.

The novelty of this study is that it is the first to analyze the routine pathology reporting of the application of pNETs nomenclature and sampling methods on a population base including over 3000 patients. Therefore, this analysis is less sensitive to confounding factors caused by a single pathology department or pathologist which might lead to deviation from the WHO classification. Moreover, we have assessed trends from 2003 to 2012, and this still has implications for the current practice because the WHO 2015 classification of pNETs reflects that of 1999 and 2004 very closely.

There are some limitations to this analysis. One of these is the use of summaries (conclusions) of pathology reports. In some cases, the pathologist may elaborate on a diagnosis in the microscopy section of the body text of the pathology report but does not include this in the summary text. Also we excluded pure SCLC diagnoses in this study, as SCLC cases are often diagnosed by pathologists and this diagnosis is frequently encountered by physicians, thus non-WHO nomenclature in this category is less likely. Another limiting factor is that PALGA does not register direct contact (e.g. face-to-face or telephone conversation) between pathologists and physicians in cases where issues regarding classification may have been discussed. Finally, the retrieved pathology conclusion may represent a slight underestimation of the total amount of established conclusions as cases with uncertain lung origin were excluded.

In summary, in 15% of pNETs other than SCLC, the nomenclature in the conclusion of pathology reports deviated from that advocated by the WHO in a population based study in the Netherlands. Our results suggest that usage of non-WHO nomenclature for any reason should be avoided and equivocal cases should be discussed in a multidisciplinary meeting with the physician(s) and pathologist(s). Future research should evaluate the contribution of non-surgical obtained biopsies in the establishment of pNET diagnosis, because biopsies are used often in the daily pathology practice. Reduction of non-WHO classification nomenclature might be obtained by the use of synoptic reporting, which might stimulate pathologists to restrain to the advised nomenclature.

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Supplemental material

Explanation on formation of Table S5.1 and clustering of conclusions

Nomenclature: all diagnoses were compared with the 2015 WHO manual for usage of nomenclature. Cases with non-WHO nomenclature are presented for each category in the top.

Clustering: after screening of 3216 conclusions, 75 different diagnoses were retrieved. Of these, 55 (from 3052 conclusions) were certain final diagnoses and 20 (from 164 conclusions) described an uncertain (differential) diagnosis. After applying the proposed clustering criteria (Table 5.2), 1086 diagnoses could be clustered into carcinoid, 1316 into high-grade neuroendocrine carcinoma, and 624 as LCC/NSCLC/carcinoma with neuroendocrine features/differentiation. One diagnosis could not be clustered and was grouped as neuroendocrine tumor NOS (26 cases) (Table S5.1). The uncertain diagnoses (164 cases) could be classified into three major categories including 1) carcinoid vs. neuroendocrine carcinoma (NEC) (38 cases), 2) LCNEC vs. SCLC (79 cases), and 3) LCC/NSCLC/carcinoma with neuroendocrine features/differentiation vs. NEC (35 cases). Twelve cases could not be clustered. (Table S5.1).

Table S5.1 Overview of all diagnoses retrieved from the pathology report conclusions

Retrieved diagnosis	Number	Non-WHO nomenclature	Clustering performed after screening (criteria see Table 5.2)
	N	*Yes/No*	Category
Low grade/well diff. neuroendocrine tumor/ca. (carcinoid)	34	Yes	
Low grade neuroendocrine tumor/ca.	10	Yes	
Neuroendocrine tumor/carcinoid	6	Yes	
Well diff. neuroendocrine tumor/ca.	4	Yes	
Neuroendocrine tumor grade I	6	Yes	
Well differentiated carcinoid	3	Yes	
Highly diff. neuroendocrine tumor	1	Yes	Carcinoids
Carcinoid grade I	1	Yes	
Low grade carcinoid	1	Yes	
Neuroendocrine tumor grade II	7	Yes	
Intermediate diff. neuroendocrine ca.	5	Yes	
Typical carcinoid	470	No	
Carcinoid; no atypical features	21	No*	
Atypical carcinoid	240	No	
Carcinoid (NOS)	250	No*	
Carcinoid; unsure if typical/atypical	25	No*	
Combined carcinoid	2	No	
Neuroendocrine tumor (NOS)	26	Yes	Neuroendocrine tumors
Non-small cell neuroendocrine ca.	47	Yes	
Poorly diff. neuroendocrine non-small cell ca.	11	Yes	
High-grade neuroendocrine ca, non-small cell	6	Yes	
Neuroendocrine ca.(NOS)	102	Yes	
High-grade neuroendocrine tumor/ca.	42	Yes	High-grade neuroendocrine carcinomas
Poorly diff. neuroendocrine ca.	36	Yes	

Neuroendocrine ca., intermediate cell type	18	Yes	
Large cell neuroendocrine ca.	800	No	
Non-small cell, possible large cell neuroendocrine ca.	14	No**	
Combined large cell neuroendocrine ca. / AdC	26	No	
Combined large cell neuroendocrine ca. / SqCC	24	No	
Combined large cell neuroendocrine ca. (other)	4	No	
Combined large cell neuroendocrine ca. / Basaloid ca.	1	No	
Large cell ca., neuroendocrine morphology	2	No	High-grade neuroendocrine carcinomas
Combined SCLC	90	No	
Combined large cell neuroendocrine ca. / SCLC	93	No	
Ca. neuroendocrine features	25	Yes	
Poorly diff. carcinoma neuroendocrine features	27	Yes	
High-grade carcinoma, endocrine features	1	Yes	
Ca. neuroendocrine diff.	11	Yes	
Poorly diff. ca. neuroendocrine diff.	13	Yes	
Ca. with AdC, SqCC and neuroendocrine component	4	Yes	
SqCC with neuroendocrine component	1	Yes	
Large cell ca. neuroendocrine features	112	No	
Non-small cell ca. neuroendocrine features	57	No	
Poorly diff. large cell ca. neuroendocrine features	49	No	Carcinoma (large/non-small cell)
Poorly diff. non-small cell ca. neuroendocrine features	20	No	with
AdC neuroendocrine diff.	78	No	Neuroendocrine features or
AdC neuroendocrine features	52	No	differentiation
SqCC neuroendocrine features	15	No	
SqCC neuroendocrine diff.	22	No	
Large cell ca. neuroendocrine diff.	49	No	
Non-small cell ca. neuroendocrine diff.	36	No	
Poorly diff. large cell ca. neuroendocrine diff.	14	No	
Poorly diff. non-small cell ca. neuroendocrine diff.	15	No	
Poorly diff. large cell ca., IHC neuroendocrine features	16	No	
Non-small cell ca. IHC neuroendocrine diff.	7	No	
High-grade neuroendocrine ca. doubt LCNEC or SCLC	38	-	
SCLC or neuroendocrine ca.	19	-	Uncertain diagnoses
Poorly diff. neuroendocrine ca; doubt LCNEC or SCLC	22	-	
Neuroendocrine tumor: SCLC or (atypical) carcinoid	16	-	
Neuroendocrine carcinoma: LCNEC or atypical carcinoid	16	-	
Neuroendocrine carcinoma or carcinoid	4	-	
Carcinoid(atypical) or neuroendocrine ca.	2	-	
Large cell ca. neuroendocrine features or LCNEC	10	-	
Non-small cell ca. neuroendocrine features or neuroendocrine ca.	5	-	
Large cell ca. or LCNEC	5	-	
SCLC or non-small cell ca. neuroendocrine diff.	6	-	Uncertain diagnoses
AdC neuroendocrine features or LCNEC	4	-	
AdC neuroendocrine features or neuroendocrine ca.	3	-	
Ca. neuroendocrine features or neuroendocrine ca.	1	-	
SCLC, LCNEC or large cell ca. neuroendocrine features	1	-	
AdC neuroendocrine features or carcinoid	6	-	
Non-small cell ca. neuroendocrine features or carcinoid	1	-	
Non-small cell ca. or neuroendocrine tumor	2	-	
NSCLC neuroendocrine diff. or adenoid cystic carcinoma	1	-	
Carcinoid or hamartoma	2	-	

* Correct in WHO 2004 manual; ** Correct in the 2015 WHO manual

Abbreviations: TC, typical carcinoid; AC, Atypical carcinoid; Diff, differentiation; Ca, carcinoma; NOS , not otherwise specified; Comb, combined; NEC, Neuroendocrine carcinoma; SqCC, squamous cell carcinoma; AdC, Adenocarcinoma; SCLC, Small cell lung carcinoma; NSCLC, non-small cell lung cancer; LCC, large cell carcinoma; NE, neuroendocrine; IHC, immunohistochemical

Table S5.2A Results of an online questionnaire on interpretation of (non) WHO-nomenclature diagnoses by a panel of physicians and pathologists

Retrieved Carcinoid diagnoses	WHO 2004 diagnoses						
	Typical carcinoid	*Atypical carcinoid*	*Carcinoid (NOS)*	*LCNEC (combined)*	*NSCLC, IHC neuroendocrine diff.*	*SCLC (combined)*	*Don't know*
Carcinoid							
Pathologist	9 (47)	-	8 (42)	-	-	-	2 (11)
Physician	9 (26)	-	22 (63)	-	-	-	4 (11)
Carcinoid, uncertain typical/atypical							
Pathologist	-	3 (16)	13 (68)	-	-	-	3 (16)
Physician	-	5 (14)	23 (66)	-	1 (3)	-	6 (17)
Carcinoid; no necrosis or atypical features							
Pathologist	18 (95)	-	1 (5)	-	-	-	-
Physician	28 (80)	-	7 (20)	-	-	-	-
Neuroendocrine tumor							
Pathologist	1 (5)	-	3 (16)	-	-	1 (5)	14 (74)
Physician	1 (3)	-	1 (3)	7 (20)	3 (9)	-	23 (65)
Neuroendocrine tumor grade I							
Pathologist	15 (79)	-	-	-	-	-	4 (21)
Physician	11 (31)	-	2 (6)	3 (9)	-	-	19 (54)
Neuroendocrine tumor grade II							
Pathologist	-	13 (68)	-	-	-	-	6 (32)
Physician	1 (3)	11 (31)	1 (3)	3 (9)	-	-	19 (54)
Neuroendocrine tumor/carcinoid							
Pathologist	5 (26)	-	9 (46)	-	-	-	5 (26)
Physician	4 (11)	-	21 (60)	1 (3)	-	-	9 (26)
Low grade/well diff. neuroendocrine tumor/carcinoma (carcinoid)							
Pathologist	8 (42)	2 (11)	7 (36)	-	-	-	2 (11)
Physician	15 (43)	-	14 (40)	1 (3)	-	-	5 (14)
Low grade neuroendocrine tumor/carcinoma							
Pathologist	2 (11)	2 (11)	8 (42)	-	-	-	7 (36)
Physician	2 (6)	-	9 (26)	5 (14)	4 (11)	-	15 (43)

Table S5.2A (continued)

Retrieved Carcinoid diagnoses	WHO 2004 diagnoses						
	Typical carcinoid	*Atypical carcinoid*	*Carcinoid (NOS)*	*LCNEC (combined)*	*NSCLC, IHC neuroendocrine diff.*	*SCLC (combined)*	*Don't know*
Physician	1 (3)	-	6 (17)	10 (29)	5 (14)	1 (3)	12 (34)
Intermediate differentiated neuroendocrine carcinoma							
Pathologist	-	3 (16)	-	2 (11)	-	1 (5)	13 (68)
Physician	-	1 (3)	2 (6)	8 (23)	6 (17)	-	18 (51)

Abbreviations: NOS, not otherwise specified; IHC, immunohistochemical; LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma SCLC, small cell lung carcinoma; WHO, World Health Organization

Table S5.2B Results of an online questionnaire on interpretation of (non) WHO-nomenclature diagnoses by a panel of physicians and pathologists

Retrieved neuroendocrine carcinoma diagnoses	WHO 2004 diagnoses						
	Typical carcinoid	*Atypical carcinoid*	*Carcinoid (NOS)*	*LCNEC (combined)*	*NSCLC, IHC neuroendocrine diff.*	*SCLC (combined)*	*Don't know*
Combined LCNEC/AdC							
Pathologist	-	-	-	15 (78)	2 (11)	-	2 (11)
Physician	-	-	-	13 (37)	17 (49)	-	5 (14)
Combined LCNEC/SqCC							
Pathologist	-	-	-	14 (73)	3 (16)	-	2 (11)
Physician	-	-	-	14 (40)	15 (43)	-	6 (17)
NSCLC, possible LCNEC							
Pathologist	-	-	-	5 (26)	4 (21)	-	10 (53)
Physician	-	-	-	12 (34)	15 (43)	-	8 (23)
NSCLC, neuroendocrine carcinoma							
Pathologist	-	-	-	14 (74)	1 (5)	-	4 (21)
Physician	-	-	-	13 (37)	14 (40)	1 (3)	7 (20)
Poorly differentiated neuroendocrine NSCLC							
Pathologist	-	-	-	12 (63)	3 (16)	-	4 (21)
Physician	-	-	-	10 (29)	22 (63)	-	3 (9)
High-grade neuroendocrine carcinoma, NSCLC							
Pathologist	-	-	-	17 (89)	-	-	2 (11)
Physician	-	-	-	11 (31)	13 (38)	-	11 (31)
Neuroendocrine carcinoma							
Pathologist	1 (5)	-	-	7 (37)	-	2 (11)	9 (47)
Physician	-	-	1 (3)	16 (46)	3 (8)	2 (6)	13 (37)
Neuroendocrine carcinoma, intermediate cell type							
Pathologist	-	2 (11)	-	2 (11)	1 (5)	2 (11)	12 (62)
Physician	-	1 (3)	2 (6)	7 (20)	5 (14)	-	20 (57)
High-grade neuroendocrine tumor/carcinoma							
Pathologist	-	-	-	11 (58)	-	2 (11)	2 (11)
Physician	-	1 (3)	1 (3)	11 (31)	3 (9)	2 (6)	17 (48)
Poorly differentiated neuroendocrine carcinoma							
Pathologist	-	-	-	7 (37)	1 (5)	2 (11)	9 (47)
Physician	-	-	-	12 (34)	8 (23)	1 (3)	14 (40)

Abbreviations: NOS , not otherwise specified; IHC, immunohistochemical; LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma SCLC, small cell lung carcinoma; WHO, World Health Organization; AdC, adenocarcinoma; SqCC, squamous cell carcinoma

Table S5.2C Results of an online questionnaire on interpretation of (non) WHO-nomenclature diagnoses by a panel of physicians and pathologists

Retrieved carcinoma with neuroendocrine features/differentiation diagnosis	WHO 2004 diagnoses						
	Typical carcinoid	*Atypical carcinoid*	*Carcinoid (NOS)*	*LCNEC (combined)*	*NSCLC, IHC neuroendocrine diff.*	*SCLC (combined)*	*Don't know*
LCC, neuroendocrine features							
Pathologist	-	-	-	4 (21)	12 (63)	-	3 (16)
Physician	-	-	-	18 (51)	15 (43)	-	2 (6)
LCC, neuroendocrine differentiation							
Pathologist	-	-	-	4 (21)	12 (63)	-	3 (16)
Physician	-	-	-	18 (51)	15 (43)	-	2 (6)
Poorly differentiated LCC, neuroendocrine features							
Pathologist	-	-	-	3 (16)	12 (63)	-	4 (21)
Physician	-	-	-	19 (54)	14 (40)	-	2 (6)
Poorly differentiated LCC, neuroendocrine differentiation							
Pathologist	-	-	-	4 (21)	11 (58)	-	4 (21)
Physician	-	-	-	15 (43)	18 (51)	-	2 (6)
Poorly differentiated LCC, IHC neuroendocrine features							
Pathologist	-	-	-	-	18 (95)	-	1 (5)
Physician	-	-	-	9 (26)	25 (71)	-	1 (3)
NSCLC, neuroendocrine features							
Pathologist	-	-	-	2 (10)	14 (74)	-	3 (16)
Physician	-	-	1 (3)	4 (11)	29 (83)	-	1 (3)
NSCLC, neuroendocrine differentiation							
Pathologist	-	-	-	1 (5)	17 (90)	-	1 (5)
Physician	-	-	-	2 (6)	29 (83)	-	4 (11)
Poorly differentiated NSCLC, neuroendocrine features							
Pathologist	-	-	-	1 (5)	14 (74)	-	4 (21)
Physician	-	-	-	4 (11)	30 (86)	-	1 (3)
Poorly differentiated NSCLC, neuroendocrine differentiation							
Pathologist	-	-	-	2 (11)	13 (68)	-	4 (21)
Physician	-	-	-	6 (17)	28 (80)	-	1 (3)
NSCLC, IHC neuroendocrine differentiation							
Pathologist	-	-	-	1 (5)	17 (90)	-	1 (5)
Physician	-	-	-	2 (6)	33 (94)	-	-

Table S5.2C (continued)

Retrieved carcinoma with neuroendocrine features/differentiation diagnosis	WHO 2004 diagnoses						
	Typical carcinoid	*Atypical carcinoid*	*Carcinoid (NOS)*	*LCNEC (combined)*	*NSCLC, IHC neuroendocrine diff.*	*SCLC (combined)*	*Don't know*
Physician	-	-	-	4 (11)	28 (80)	-	3 (9)
SqCC, neuroendocrine differentiation							
Pathologist	-	-	-	1 (5)	16 (84)	-	2 (11)
Physician	-	-	-	4 (11)	30 (86)	-	1 (3)
AdC, neuroendocrine differentiation							
Pathologist	-	-	-	-	17 (90)	-	2 (10)
Physician	-	-	-	2 (6)	32 (91)	-	1 (3)
AdC, neuroendocrine features							
Pathologist	-	-	-	-	15 (79)	-	4 (21)
Physician	-	-	-	2 (6)	32 (91)	-	1 (3)
Carcinoma, neuroendocrine features							
Pathologist	-	-	-	1 (5)	13 (68)	-	5 (26)
Physician	1 (3)	0	1 (3)	7 (20)	14 (40)	-	12 (34)
Carcinoma, neuroendocrine differentiation							
Pathologist	-	-	-	3 (16)	10 (53)	-	6 (32)
Physician	-	-	1 (3)	4 (11)	17 (49)	-	13 (37)
Poorly differentiated carcinoma, neuroendocrine features							
Pathologist	-	-	-	2 (11)	12 (63)	-	5 (26)
Physician	-	1 (3)	-	5 (14)	20 (57)	-	9 (26)
Poorly differentiated carcinoma, neuroendocrine differentiation							
Pathologist	-	-	-	2 (11)	9 (47)	-	8 (42)
Physician	-	1 (3)	-	7 (20)	21 (60)	-	6 (17)

Abbreviations: NOS , not otherwise specified; IHC, immunohistochemical; LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma SCLC, small cell lung carcinoma; WHO, World Health Organization; AdC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma

Chapter 6

Reporting of World Health Organization classification diagnostic criteria for large cell neuroendocrine carcinoma in pathology reports: a comprehensive analysis of daily practice

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Abstract

Previously we have shown that application of the World Health Organization (WHO) classification nomenclature for pulmonary large cell neuroendocrine carcinoma (LCNEC) is subjected to significant variation. Here we have analyzed application of the WHO criteria in LCNEC pathology reports.

Digital summaries of histologically established LCNEC diagnoses retrieved from the Netherlands Pathology Registry (PALGA) and Cancer Registry (2003-2012) were identified (n=1180); hardcopies of full reports were retrieved (n=882) to screen for WHO criteria. Independent, blinded pathology review was performed on selected cases (n=210, 24%) to evaluate presence of WHO criteria in original tumor samples.

Reports retrieved described 438 resection, 235 needle biopsy and 205 trans/endobronchial biopsy (EBB/TBB) specimens. Mitosis was described in 71%, necrosis in 62%, neuroendocrine morphology in 54% and neuroendocrine markers in 92% of reports, respectively. Only 14% of all reports described a mitotic index. The criteria for LCNEC were described in only 28% of reports, more often in resection (40%) than in needle biopsy (23%) and EBB/TBB specimen (12%, both $P<0.001$). LCNEC was confirmed in 146/210 (70%) of pathology reviewed cases. All diagnostic criteria for LCNEC were identified in 79% (n=38) of biopsy samples in which the original pathology reports described all WHO criteria. Similarly, all criteria were identified in 68% (n=110) of tumor samples in which the original pathology report did not describe all WHO criteria, not statistically different (79% *versus* 68%, $P=0.13$).

In only 28% of all LCNEC diagnoses established, all diagnostic WHO criteria were described in the original pathology report. No difference in identification of WHO criteria was identified by panel review of original tumor slides from pathology reports (not) describing all WHO criteria. Summarizing identified WHO criteria should be encouraged and might be helpful for clinicians for interpretation.

Introduction

Pulmonary neuroendocrine tumors are separated into typical carcinoid, atypical carcinoid, large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC)[1,2]. The incidence of carcinoids and LCNEC is low, ranging from 100-150 patients per year in the Netherlands[3,4]; however, it has been shown that these entities are increasingly diagnosed in the Netherlands and worldwide[5-9].

LCNEC was initially recognized in 1991 and the diagnostic criteria include a neuroendocrine growth pattern (organoid nesting, trabeculae, palisading cells or rosettes), a high mitotic index (>10 2mm^2), central necrosis and a large cell type (i.e. with abundant cytoplasm or conspicuous nucleoli)[10,11]. These criteria have been used in the WHO classification of 1999, 2004 and recently the 2015 classification[1,2,12]. In the current diagnostic process the diagnostic classification of LCNEC is an umbrella term; it encompasses cases which overlap with i) small cell carcinoma, ii) non-small cell carcinoma with neuroendocrine features and carcinoid tumor with >10 mitosis per 2mm^2 [13].

Although the diagnostic criteria for LCNEC are well described several limitations have been reported[14,15]. The diagnostic separation of LCNEC from atypical carcinoid can be problematic as counting of mitosis can be difficult and diagnostic variation among pathologists is not uncommon[16-20]. Also cell size, a cytological diagnostic criterion that helps to separate LCNEC from SCLC may overlap in these entities[17,21-23]. Finally, the neuroendocrine morphology and neuroendocrine immunohistochemical staining observed in LCNEC can be heterogeneous (WHO 2015), leading to a possible non-small cell carcinoma (NSCLC) diagnosis. Accurate and precise classification of pulmonary neuroendocrine tumors is important as the prognosis of carcinoids is better than for LCNEC and SCLC[24-28]. Also, systematic treatment of LCNEC is different from carcinoids and disputed for SCLC and NSCLC[25,29-32].

Previously we have shown that application of the WHO classification nomenclature in pulmonary neuroendocrine tumors significantly varies in daily pathology practice and that variation was most frequently found in high-grade tumor diagnoses[5]. The WHO 2004 classification did not allow a diagnosis of LCNEC on a biopsy specimen[12]. With the WHO 2015 classification, a diagnosis 'NSCLC, possible LCNEC' can be established when neuroendocrine morphology is observed in a biopsy specimen[1]. We recently observed that LCNEC has been increasingly diagnosed on biopsy specimens, even before the WHO 2015 classification was implemented[5]. Therefore, we analyzed whether pathologists

adhere to the WHO criteria for the diagnosis of LCNEC, by analyzing notification of these criteria in pathology reports on a population basis in the Netherlands between 2003 and 2012 and by performing pathology revision on a subgroup of these tumors.

Materials and methods

Data sources

For this population based study, all pathology data were retrospectively retrieved from the Netherlands Pathology Registry, PALGA[33] (the nationwide network and registry of histo- and cytopathology in the Netherlands). Pathology data retrieved from the pathology database were combined with data from the Netherlands Cancer Registry to confirm lung cancer diagnosis.

Case selection

The pathology database was screened for digital written conclusions of pathology reports containing any diagnosis corresponding with LCNEC diagnosed between 01-01-2003 and 31-12-2012 according to previously described search criteria[5]. Because the cancer registry uses several codes that may include patients with LCNEC, different codes were applied to include all LCNEC patients including the international classification of disease 3rd edition code M8013 (LCNEC), M8246 (neuroendocrine carcinoma) and non-small cell lung carcinoma with immunohistochemical neuroendocrine differentiation (M8574). Data from the registries were then combined and patients with a digital written conclusion containing any diagnosis corresponding to LCNEC, NSCLC neuroendocrine carcinoma, or NSCLC favor LCNEC were selected. Additionally, written digital summaries of all cancer pathology diagnoses including those 7 years before/after original diagnosis were retrieved from the pathology database.

Screening of digital pathology summaries

Written digital pathology summaries were screened by a single reader (JD) and in cases of doubt RvS was consulted for advice. Data on sampling location (e.g. lung, liver or lymph node), sampling technique (non-surgically obtained biopsy, cytology or resection specimen), and final diagnosis were extracted. If a patient had multiple corresponding diagnoses described in the digital pathology reports, the diagnosis corresponding with the largest tissue specimen (resection>biopsy>cytology) or a second opinion diagnosis, was selected by the screener as previously described[5]. Subsequently, anonymized hardcopies of original full pathology reports describing a diagnosis of LCNEC on a

histopathological specimen, excluding autopsies, were requested from all pathology departments in the Netherlands.

Pathology report screening for mandated diagnostic LCNEC criteria (World Health Organization 2015)

All retrieved full pathology reports were manually screened (JD) and data extracted included: diagnosis, when multiple diagnoses were described the possible differential diagnoses, sampling technique(s), sampling location, and type of pathology report either as original evaluation or as second opinion evaluation. The reports were point by point screened for the following WHO 2015 criteria for LCNEC i) neuroendocrine growth pattern (organoid nesting, trabeculae, palisading cells or rosettes), ii) mitotic index ($2mm^2$/10 high power fields) or described as none/few/many if no index was described, iii) necrosis (none, dotlike/focal as in atypical carcinoid or abundant/central), and IV) staining of immunohistochemical (IHC) neuroendocrine markers including CD56/NCAM, chromogranin-A and/or synaptophysin. Cytological features for NSCLC were not included in this analysis.

Evaluation of mandated WHO criteria retrieved from the LCNEC pathology reports

Complete description of the WHO criteria was defined as i) any neuroendocrine growth pattern, ii) a mitotic index ($2mm^2$ or >10 mitosis per 10 high power fields) or description of abundant tumor necrosis and iii) any positive neuroendocrine IHC marker. Description of ''vague'' terminology was scored by RvS, i.e. written terminology that was not according to the WHO, but could be interpreted as such. In a secondary analysis, this vague terminology was included for scoring of WHO criteria (Table 6.1).

Panel consensus pathology revision for diagnostic WHO criteria

Tumor histology slides were collected and included at least one immunohistochemical neuroendocrine stain (CD56/NCAM, chromogranin-A or synaptophysin) and a hematoxylin and eosin (HE) stained slide. Review was performed by three pathologists (RvS, MdB and ET) as previously published[32]. All HE slides were evaluated at the multi-head microscope; information of IHC expression patterns was assessed previously and provided to the reviewers. All WHO 2015 criteria for neuroendocrine lung tumors were evaluated by means of a structured report. Consensus was established when at least two pathologists agreed on the diagnosis.

Table 6.1 Criteria used to analyze the WHO 2015 criteria description in full pathology reports

Criteria	WHO 2015 criteria for LCNEC	Analyzed WHO 2015 criteria (1)	Analyzed WHO 2015 criteria including *"vague terminology" (2)*
Neuroendocrine morphology	Organoid nesting, palisading, rosettes or trabeculae	Description of organoid nesting, palisading, rosettes or trabeculae *OR "neuroendocrine morphology" observed described by the pathologist*	Description of Organoid nesting, palisading, rosettes or trabeculae *OR "neuroendocrine morphology" observed described by the pathologist* *OR "vague morphology*"*
Mitotic index	≥11 or greater per 2mm² (10 HPF)	≥11 or greater per 2mm² (10 HPF) *OR necrosis (often large zones)*	≥11 or greater per 2mm² (10 HPF) *OR "vague mitosis[i]"* *OR necrosis (often large zones)*
Necrosis	Necrosis (often large zones)	See above.	See above.
Neuroendocrine differentiation	Positive immunohistochemical staining for one or more NE markers and/or NE granules by electron microscopy	Positive immunohistochemical staining for one or more NE markers.	Positive immunohistochemical staining for one or more NE markers.
Cytological Features	Cytological features of a non-small cell carcinoma	Not assessed	Not assessed

*Vague morphology included ambiguous description of neuroendocrine morphology as assessed by JD and RvS. [i] Vague mitosis included ambiguous description of mitosis that lacked a mitotic index as assessed by JD and RvS

Statistical analysis

The chi-square or fisher exact test was used to compare categorical data. *P*-values were tested two sided and *p*-values <0.05 were considered statistically significant. SPSS (version 22.0 for Windows (SPSS Inc. Chicago. IL)) was used for all statistical analysis.

Results

Overview of LCNEC cases diagnosed in the Netherlands (2003-2012)

From the cancer registry database, data from 1627 unique patients were retrieved; similarly, data from 1172 unique patients were retrieved from the pathology database. After the data was merged, 2066 unique patients were identified of whom 175 were excluded because of a non-lung cancer diagnosis. Subsequent evaluation of the patients' pathology history by manual screening of digital written summaries identified 1105 patients with LCNEC and 1416 unique digital summaries describing of pathology reports describing LCNEC. After applying the exclusion criteria, 1180 digital summaries describing a histopathological LCNEC diagnosis were selected and hardcopies of the complete pathology reports were requested. In total 882 (75%) hardcopies were received for WHO criteria assessment. Finally, from 210 pathology reports the corresponding original tumor slides were obtained and panel-consensus review was performed. (Flowchart Figure 6.1).

Establishment of LCNEC diagnosis

The diagnosis of 1416 identified written digital conclusions was LCNEC (87%), combined squamous carcinoma-LCNEC (2%) and combined adenocarcinoma-LCNEC (2%). In 36% the diagnosis was established on a resection specimen, in 47% on a biopsy specimen and in 15% on a cytological specimen (Table 6.2).

Analysis of notification of mandated WHO criteria in pathology reports describing LCNEC

Notification of all WHO–criteria, including a mitosis index and necrosis, was described in only 5% (n=46) of analyzed LCNEC pathology reports. Therefore, the mitotic index (>10 per $2mm^2$ or 10 high power fields) *or* necrosis (abundant) was scored as appropriate for high-grade tumor classification. Using these adjusted criteria, notification of LCNEC WHO criteria increased from 5% to 29% (n=225).

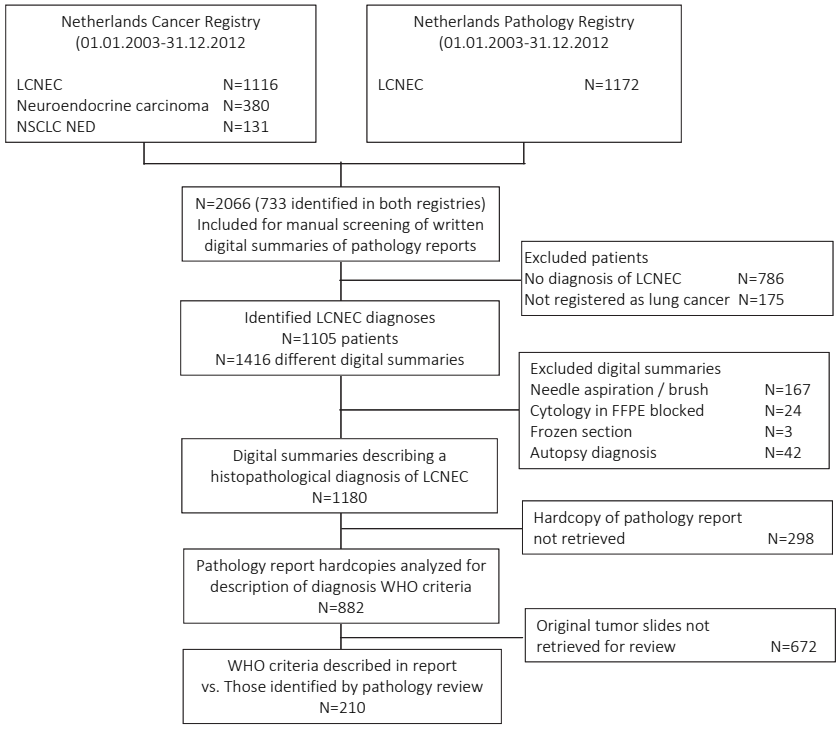

Figure 6.1 Screening and selection of registries for diagnosis of LCNEC and subsequent retrieval of hardcopies of LCNEC pathology reports
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; NSCLC NED, non-small cell lung carcinoma with neuroendocrine immunohistochemical differentiation; WHO, world health organization

These latter criteria were further used in to describe the results. Complete notification on the WHO criteria did not change over time (≤2007 28% (n=84) *versus* >2007 30% (n=171) *P*=0.54), (Figure 6.2a) and was found significantly more frequent in pathology reports describing a resection specimen (40%) compared to core needle biopsy specimen (23%, *P*<0.001) and endobronchial/trans-bronchial biopsy (EBB/TBB) specimen (12%, *P*<0.001) (Figure 6.2b). The type of hospital establishing the LCNEC diagnosis did not correlated with notification of WHO criteria (i.e. academic hospital [31%] *versus* non-academic [28%], *P*=0.40, Figure 6.2c). We observed a modest variance in complete notification of the WHO criteria when anonymized pathology laboratories were analyzed on an individual level (Figure 6.3a). A higher volume of LCNEC diagnoses increased notification of WHO criteria slightly (≤18 reports [22%] *versus* >18 reports [32%], *P*=0.006, Figure 6.2d). When vague terminology was included in the assessment of WHO criteria, than 42% of pathology reports described all criteria. For example, vague

description of a WHO criteria included description of “peripheral alignment of tumor cells” to describe palisading cells for neuroendocrine morphology, and “many mitoses” to describe mitosis but without a mitotic index. Results regarding effects over time, per specimen type, and for type of hospital/volume for notification on criteria that included vague described terminology are presented in Figure 6.2 e-h and Figure 6.3b.

Table 6.2 Selected written digital summaries describing LCNEC retrieved from the pathology database

	Total cases		
	All	*Histology*	*Analyzed reports*
Variables	*N (%)*	*N (%)*	*N (%)*
LCNEC diagnosis	1416	1180	882
LCNEC*	1239 (87)	1042 (88)	736 (83)
Combined LCNEC/SqCC	23 (2)	23 (2)	20 (2)
Combined LCNEC/AdC	27 (2)	27 (2)	21 (2)
Combined LCNEC (NOS)	11 (1)	11 (1)	5 (1)
NSCLC or high grade NEC, favor/possible LCNEC	116 (8)	77 (7)	71 (8)
Original diagnosis other than LCNEC, revised as LCNEC	-	-	27 (3)
Diagnosis other than LCNEC in pathology report	-	-	2 (1)
Sampling technique			
Resected tissue	507 (36)	507 (43)	438 (50)
Needle biopsy	275 (19)	275 (23)	235 (26)
EBB/TBB or NOS	398 (28)	398 (34)	204 (23)
Cytology, including brush, FNA or aspirate	191 (15)	-	-
Autopsy	42 (3)	-	-
Frozen section for surgical margin	3 (0)	-	-
Not described	-	-	5 (1)

* Including diagnosis with varying nomenclature including LCNEC and NSCLC neuroendocrine carcinoma
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinoma; NOS, not otherwise specified; NEC, neuroendocrine carcinoma; EBB/TBB, endobronchial or trans bronchial biopsy specimen; FNA, fine needle aspiration

Description of neuroendocrine morphology, mitosis and necrosis in pathology reports

An overview of the frequency of notification of the WHO criteria is given in Table 6.3. Notification of neuroendocrine morphology was present in 54% of analyzed reports and generally described without details on the subtype of morphology (e.g. organoid nesting or rosettes). Notification on neuroendocrine morphology increased to 61% when vague terminology was included. On core needle biopsy specimens, frequency of notification on neuroendocrine morphology was relatively similar to that of resection specimens (60% *versus* 55%, *P*=0.29) but significantly higher than in EBB/TBB specimens (40%, *P*<0.001). Although mitoses were described regularly (71%), many descriptions of mitoses included vague descriptions such as “numerous mitosis or mitosis are frequently observed”.

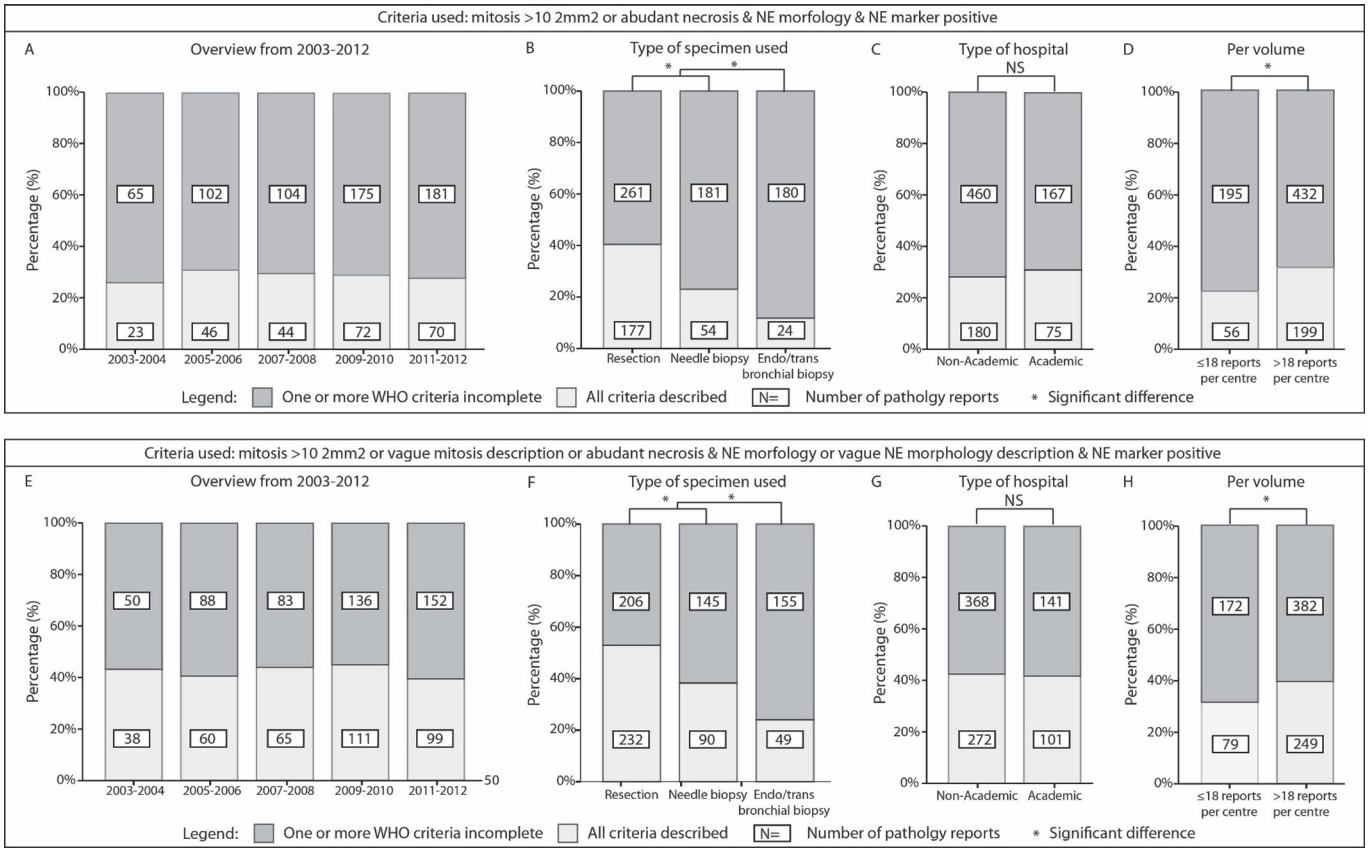

Figure 6.2 **A-D**) Overview on description of the WHO 2015 criteria in analyzed pathology reports of LCNEC cases and **E-H**) description of the WHO 2015 criteria including vague mitosis and NE morphology descriptions

Abbreviations: WHO, World Health Organization; LCNEC, large cell neuroendocrine carcinoma; NE, neuroendocrine; NS, not statistically significant different

Table 6.3 Characteristics of retrieved LCNEC pathology reports screened for description of the diagnostic WHO criteria

Variable	Total*	Sampling technique			Second opinion Reports
		Resection	Needle biopsy	EBB/TBB	
	N (%)	N (%)	N (%)	N (%)	N (%)
Number of analyzed pathology reports	882 (100)	438 (50)	235 (27)	204 (23)	82 (9)
Diagnosis					
LCNEC	736 (82)	376 (86)	197 (84)	159 (78)	66 (81)
Combined SqCC or AdC-LCNEC	46 (5)	36 (8)	7 (3)	3 (2)	6 (7)
Carcinoma, favor LCNEC	71 (8)	16 (4)	24 (10)	31 (15)	9 (11)
Not a LCNEC diagnosis after second opinion	2 (1)	1 (0)	0 (0)	0 (0)	1 (1)
Diagnosis changed to LCNEC after second opinion in the pathology report	27 (3)	9 (2)	7 (3)	11 (5)	0 (0)
Mitosis described	623 (71)	332 (76)	166 (70)	123 (60)	51 (62)
>10 mitosis/10HPF or 2mm^2	127 (14)	87 (20)	27 (12)	13 (6)	8 (10)
≤10 mitosis/10HPF or 2mm^2	12 (1)	1 (0)	8 (3)	3 (2)	4 (5)
Vague description of mitoses					
Multiple/many/numerous mitoses	342 (39)	204 (47)	75 (32)	61 (30)	30 (36)
Infrequent, some, few mitoses	43 (4)	6 (1)	19 (8)	18 (9)	2 (2)
Unclassifiable	99 (11)	34 (8)	37 (16)	28 (14)	7 (9)
Necrosis described	550 (62)	342 (78)	127 (54)	80 (39)	49 (60)
Large zones, substantial, central necrosis	369 (42)	264 (60)	74 (52)	31 (15)	30 (37)
Focal, dot like, occasional necrosis	53 (6)	25 (6)	18 (8)	10 (5)	4 (5)
No necrosis	30 (3)	10 (3)	9 (4)	11 (5)	7 (9)
Unclassifiable	98 (11)	43 (10)	26 (11)	28 (14)	8 (10)
Neuroendocrine morphology	475 (54)	261 (60)	130 (55)	82 (40)	42 (51)
Nesting (organoid)	172 (20)	83 (19)	60 (26)	28 (14)	16 (20)
Trabeculae	118 (13)	82 (19)	23 (10)	13 (6)	10 (12)
Palisading cells	97 (11)	71 (16)	17 (7)	9 (4)	12 (15)
Rosettes	115 (13)	80 (18)	20 (9)	14 (7)	11 (13)
Neuroendocrine morphology (yes) in text[i]	216 (25)	106 (24)	65 (28)	44 (22)	19 (23)
"Vague" neuroendocrine morphology	163 (19)	93 (21)	41 (17)	28 (14)	11 (13)
Neuroendocrine IHC stain performed	807 (92)	388 (89)	224 (95)	192 (94)	71 (87)
3 markers tested	509 (58)	237 (54)	153 (65)	119 (58)	44 (54)
3 marker positive †	274 (54)	129 (54)	87 (57)	58 (49)	27 (61)
2 markers tested	708 (80)	343 (78)	200 (85)	163 (80)	62 (76)
2 marker positive †	556 (79)	267 (79)	167 (84)	121 (74)	50 (81)
Stained for CD56 (NCAM)	784 (89)	388 (89)	216 (92)	176 (87)	64 (78)
Positive†	691 (88)	347 (89)	189 (87)	153 (85)	57 (89)
Negative†	73 (9)	25 (6)	25 (12)	23 (13)	6 (9)
Analyzed on previously sampled tissue†	20 (3)	16 (5)	2 (1)	2 (2)	1 (2)
Stained synaptophysin	637 (73)	308 (71)	180 (77)	147 (72)	59 (72)
Positive†	533 (83)	249 (80)	164 (91)	118 (80)	53 (90)
Negative†	95 (15)	50 (16)	16 (9)	29 (20)	6 (10)
Analyzed on previously sampled tissue†	9 (2)	9 (4)	0 (0)	0 (0)	0 (0)
Stained for chromogranin-A	651 (74)	310 (71)	187 (80)	153 (75)	55 (67)
Positive†	413 (63)	188 (60)	125 (67)	100 (65)	38 (69)
Negative†	229 (35)	113 (36)	62 (33)	53 (35)	17 (31)
Analyzed on previously sampled tissue†	9 (2)	9 (4)	0 (0)	0 (0)	0 (0)

[i] In text neuroendocrine morphology presence is described but type observed is not specified. * In 5 cases the sampling method could not be retrieved, these are only presented under the total cohort. † Percentage from total number of pathology reports reporting staining for this/these marker(s)

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; IHC, immunohistochemical staining; EBB/TBB, endobronchial or trans bronchial biopsy specimen; SqCC, squamous cell carcinoma; AdC, adenocarcinoma; HPF, high power fields

The mitotic index was described in 15% of all analyzed reports and more frequently observed in resection specimens (20%) compared to needle biopsy (12%, *P*=0.001) and EBB/TBB specimens (6%, *P*=0.001). Notification on necrosis could be retrieved in 62% of pathology reports and was less frequently described in core needle biopsies (54%, *P*<0.001) and EBB/TBB (39%, *P*<0.001). A single neuroendocrine marker (i.e. CD56, synaptophysin or chromogranin-A) was performed in 92% of reports and was reported as positive for CD56 in 88%, for synaptophysin in 83% and for chromogranin-A in 63%. In 58% of retrieved pathology reports all three neuroendocrine markers and in 80% two neuroendocrine markers were described. Positive staining was reported for three markers in 54% and 79% for two markers. Frequency of reporting of three and two neuroendocrine marker staining increased over time (48% ≤2007 *versus* 63% >2007, *P*=0.001 and 76% *versus* 83%, *P*=0.02). Also, positive staining for three and two neuroendocrine marker staining increased from 41% to 59% (*P*=0.001) and from 72% to 82% (*P*=0.005), respectively. Finally, the frequency of neuroendocrine marker staining was not significantly different amongst specimen types.

Correlation of WHO criteria described in pathology report and identified in original matching tumor specimens by panel-consensus review

From 210 pathology reports, describing all WHO-criteria in 23%, the matching original tumor slides could be obtained and included 82 resection specimens, 88 needle biopsy specimens and 40 EBB/TBB specimens. In 148 (70%) of all reviewed tumors the WHO criteria were confirmed in the original diagnostic tumor slides and LCNEC was diagnosed by the panel in 146 (Table 6.4). In tumor samples of initial pathology reports having described all WHO criteria, all WHO criteria were also identified by panel review in 79% (n=38). In the absence of one or more WHO criteria reported by the original report all WHO criteria were identified in 68% (n=110) in the original tumor slides, not statistically different (79% *versus* 68%, *P*=0.13). Of all reviewed original tumor slides originally diagnosed as LCNEC, the mitosis index was lower than 10 or considered unclassifiable in 16% (n=32); this occurred less often in specimens of initial pathology reports that described all WHO criteria (4%, n=2), compared to evaluated samples from reports that did not describe all WHO criteria (18%, n=30, *P*=0.02). Necrosis was not identified in 31% (n=64) of tumor samples, this was less frequent when al WHO criteria were described in the initial pathology report (15%, n=7) *versus* reviewed tumor samples of reports that did not describe all WHO criteria (32%, n=57, *P*=0.02*).* In tumor samples from initial reports describing all WHO criteria, no LCNEC diagnoses were revised as atypical carcinoid by panel revision (0%, n=0). In 3% (n=5) of tumor samples that did not describe all WHO criteria in the original pathology report the diagnosis LCNEC was revised to atypical carcinoid (*P*=0.59).

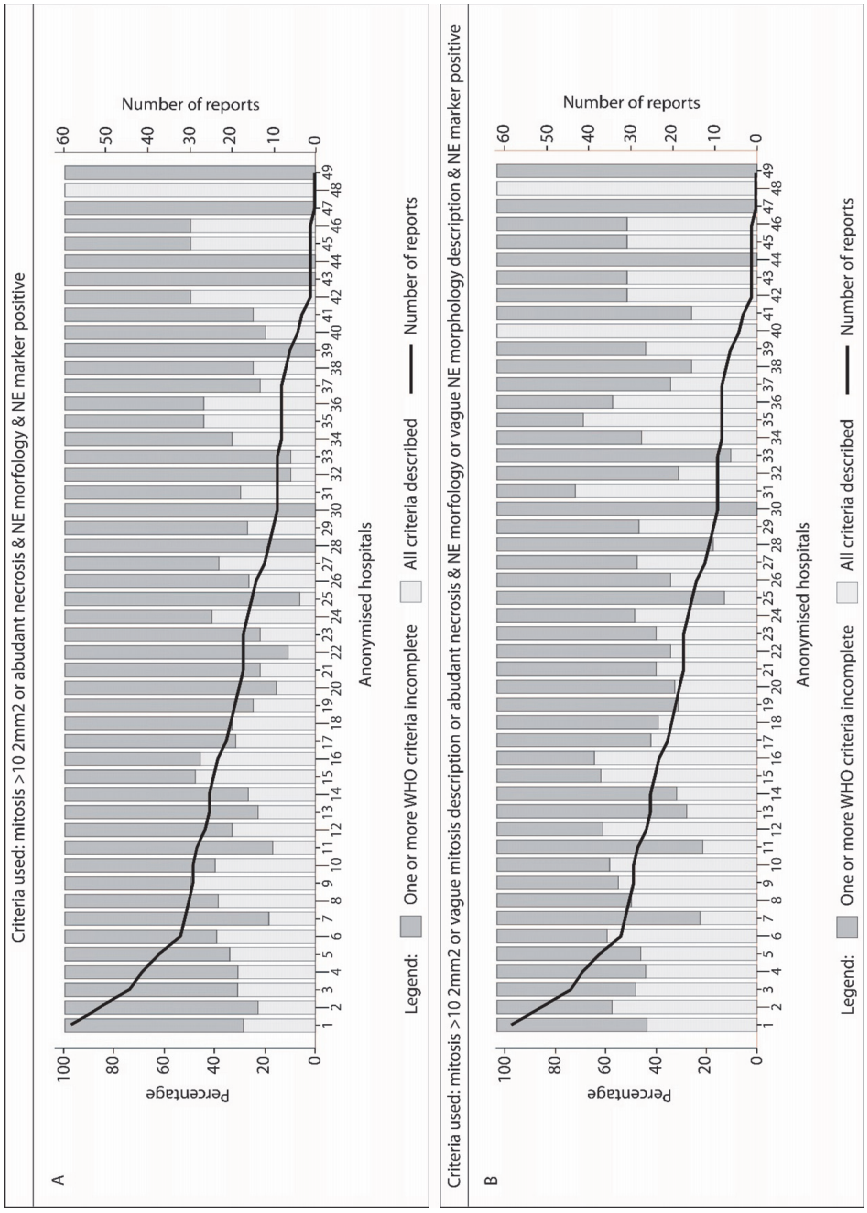

Figure 6.3 **A)** Overview on description of the WHO 2015 criteria in analyzed pathology reports of LCNEC cases depicted according to number per anonymized hospitals in the period 2003 to 2012 and **B)** description of the WHO 2015 criteria including vague mitosis or NE morphology descriptions
Abbreviations: WHO, World Health Organization; LCNEC, large cell neuroendocrine carcinoma; NE, neuroendocrine

Table 6.4 Comparison of pathology review for WHO criteria in LCNEC

	Reviewed tumors	WHO criteria described in Pathology report (1)		WHO criteria described in Pathology report (2)		*P*-value	
		Yes	*No*	*Yes*	*No*		
	N (%)	*N (%)*	*N (%)*	*N (%)*	*N (%)*	(1)	(2)
Available for consensus revision	210 (100)	48 (23)	162 (77)	65 (31)	145 (69)		
Evaluation of WHO criteria						0.13	0.30
All WHO criteria confirmed	148 (70)	38 (79)	110 (68)	49 (75)	99 (68)		
One or more WHO criteria absent	62 (30)	10 (21)	52 (32)	16 (25)	46 (32)		
High-grade disease confirmed						0.03**	0.006*
Yes	195 (93)	48 (100)	147 (91)	65 (100)	130 (90)		
No	15 (7)	0 (0)	15 (9)	0 (0)	15 (10)		
Mitosis described						0.02***	0.03
<10	14 (7)	1 (2)	13 (8)	1 (1)	13 (9)		
>10 but <30	66 (31)	13 (27)	53 (33)	20 (31)	46 (32)		
≥30	112 (53)	33 (69)	79 (49)	42 (65)	48 (70)		
Unclassifiable	18 (9)	1 (2)	17 (10)	2 (3)	16 (11)		
Necrosis described						0.02	0.08
Large zones, substantial necrosis	131 (62)	36 (75)	95 (59)	46 (71)	85 (59)		
Focal, dot like, occasional necrosis	15 (7)	5 (10)	10 (6)	6 (9)	9 (6)		
No necrosis	64 (31)	7 (15)	57 (32)	13 (20)	51 (35)		
Neuroendocrine morphology						0.14	0.28
Present	158 (75)	40 (83)	118 (73)	52 (80)	106 (73)		
Absent	52 (25)	8 (17)	44 (27)	13 (20)	39 (27)		
Neuroendocrine marker(s) positive						0.13*	0.03*
Yes	207 (99)	46 (96)	161 (99)	62 (95)	145 (100)		
No	3 (1)	2 (4)	1 (1)	3 (5)	0 (0)		

Table 6.4 (continued)

	Reviewed tumors	WHO criteria described in Pathology report (1)		WHO criteria described in Pathology report (2)		*P*-value	
		Yes	*No*	*Yes*	*No*		
	N (%)	*N (%)*	*N (%)*	*N (%)*	*N (%)*	(1)	(2)
Review consensus diagnosis						-	-
LCNEC	142 (68)	36 (75)	106 (65)	56 (74)	86 (64)		
Combined LCNEC	4 (2)	1 (2)	3 (2)	1 (1)	3 (2)		
Atypical Carcinoid	5 (2)	0 (0)	5 (3)	0 (0)	5 (4)		
SCLC	19 (9)	3 (6)	16 (10)	4 (5)	15 (11)		
NSCLC NED	19 (9)	4 (8)	15 (9)	7 (9)	12 (9)		
NSCLC NOS	4 (4)	2 (4)	2 (1)	3 (4)	1 (1)		
Not conclusive, dd LCNEC vs SCLC	5 (2)	1 (2)	4 (3)	2 (3)	3 (2)		
Not conclusive dd NSCLC NED vs LCNEC vs SCLC	8 (4)	1 (1)	7 (4)	2 (3)	6 (5)		
Other diagnosis	4 (2)	0 (0)	4 (3)	1 (1)	3 (2)		

1) Either description of mitosis (>10/10 HPF) or substantial necrosis is considered as high-grade disease. 2) Either description of mitosis (>10/10 HPF) or vague mitosis (e.g. abundant) description or substantial necrosis is considered as high-grade disease. ** Fisher Exact test. *** <10 mitosis and unclassifiable were clustered to enable analysis with Fisher Exact test.

Abbreviations: HPF, high power fields; WHO, World Health Organization; dd, differential diagnosis; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; NED, neuroendocrine differentiation; NOS, not otherwise specified.

Discussion

Between 2003 and 2012 the WHO criteria for LCNEC were often not described in original pathology reports, but this did not necessarily reflect the quality of the diagnosis. This conclusion is supported by our findings that i) in only 5% of analyzed LCNEC pathology reports all mandated WHO criteria could be identified; increasing to 29% when either the mitotic index or necrosis (large zones) was accepted to fulfil high-grade tumor classification. And ii) panel review confirmed a LCNEC diagnosis in 70% of revised cases; not different for reviewed diagnoses of pathology reports describing all WHO criteria *versus* reviewed diagnoses from pathology reports not describing all these criteria.

Absence of WHO criteria of LCNEC reported in the pathology report, occurred more often on biopsy specimen than on resection specimen. Mitosis and necrosis were less frequently described in biopsy specimen and almost never included a mitotic index. By panel review we observed that in biopsy specimen mitosis and necrosis occasionally could not be identified and that this occurred more often in revised tumor samples from pathology reports not describing all WHO criteria. Importantly, according to the current WHO classification, establishment of LCNEC is not possible in tumor specimen not fulfilling the criteria of >10 mitosis / $2mm^2$ [1]. A recent study emphasized that application of a Ki-67 proliferation index >20% might be more sensitive and specific to separate carcinoids from high-grade neuroendocrine carcinomas on biopsy specimens[34]. The importance of accurately separating carcinoids from high-grade neuroendocrine carcinomas has been emphasized by case reports of crushed biopsies taken from carcinoid tumors originally diagnosed as high-grade neuroendocrine carcinoma but revised as carcinoid on basis of a low Ki-67 proliferation index[35,36].

Incomplete description of the WHO diagnostic criteria in a pathology report does not necessarily reflect the quality of a LCNEC diagnosis; it may be caused unintentionally by differences in reporting systems or habits between different pathologists and laboratories. Alternatively, complete description may neither mean that the diagnosis (although all criteria are applied) is correct. Overall 30% of original LCNEC diagnoses were revised; this was not significantly related to the description of the WHO criteria for LCNEC. However, pathology reports with a diagnosis of LCNEC that did not describe all criteria (mainly mitosis) did include atypical carcinoid diagnoses after revision; this was not observed in reports describing all WHO criteria. A recent analysis of the WHO 2010 criteria for large cell neuroendocrine of the gastrointestinal tract (2008-2012) showed that 37% of LCNEC diagnoses were according to the 2010 criteria and accurate description of all WHO criteria was related to the patients prognosis[37].

Several pathology reports included terminology that was difficult to interpret using the diagnostic WHO criteria. Unclear terminology regularly related to description of mitosis or neuroendocrine morphology. Vague phraseology has been investigated in different circumstances; however, these might be interpreted differently by treating clinicians than by pathologists, as we and others have reported and therefore preferably remains limited in pathology reporting[38,39].

Previous studies have shown that reporting on histological tumor type, histological grade and number of lymph node metastasis is adequate in the daily pathology practice with over 90% being scored in narrative pathology reports in all cancers, and 87%-98% in lung cancer[40,41]. Here we show that reporting of the WHO 2015 criteria for LCNEC is only 5-29%, and 40% in the resection specimen. Hence, introduction of a structured diagnostic algorithm might be useful to increase description of criteria for LCNEC and other neuroendocrine lung tumors. In 2016 such a system was also advocated in Japan after analysis of pathology reporting in gastrointestinal neuroendocrine tumors and by a Delphi consensus analysis on pathology reporting[42,43]. The Netherlands Pathology registry has recently developed a surgical resection and biopsy synoptic reporting protocol for lung cancer that is implemented throughout the Netherlands in 2017. For any neuroendocrine tumor diagnosis, pathologists are automatically requested to fill in the mitotic index, amount of necrosis and for LCNEC the observed type of neuro-endocrine morphology and type of staining for neuroendocrine immunohistochemical markers.

LCNEC is known to be a difficult diagnosis with a broad differential diagnosis including carcinoid, SCLC, basaloid carcinoma or NSCLC with neuroendocrine differentiation[1,14,44]. Also, interobserver variation has been reported with (atypical) carcinoid and SCLC[13,17-19]. In a previous study including LCNEC diagnosed in the daily pathology practice, all were confirmed by pathology revision[27]. In this study we found that up to 70% of LCNEC diagnosed in daily practice could be confirmed, slightly biased due to the high frequency of biopsies included in the pathology revision.

To our opinion a straightforward description and adherence to defined criteria eases clinical decision making. Doing so, the physician has the possibility to assess the certainty with which a diagnosis is established[45]. This can be of importance when the patient's clinical presentation does not fit the diagnosis or whenever the response to therapy or prognosis is different from what the physicians is expecting[24,46]. Recent advances in the treatment of carcinoids with mTOR inhibitors, peptide receptor radionuclide therapy

(PRRT) and somatostatin analogues strengthen the need to differentiate carcinoids from high-grade neuroendocrine carcinomas such as LCNEC[25,29,47].

In conclusion, the current WHO criteria required to come to a diagnoses of LCNEC are regularly not fully described in the daily pathology practice with frequent use of vague terminology, mainly on biopsy specimens. Lack of description of WHO criteria occurs in all pathology departments and only increased slightly when the diagnosis is established more often. The diagnostic criteria for LCNEC frequently were absent in original tumor slides when assessed by panel review of originally diagnosed LCNEC. Yet, despite that up to 30% of original LCNEC diagnoses were diagnosed differently by panel review, a lack of description of the WHO criteria did not correlate with a significant higher proportion of revised diagnoses. Future studies should investigate if by synoptic reporting adherence to WHO criteria increases and if this results in a more accurate LCNEC diagnosis. Finally, additional criteria for establishment of LCNEC diagnoses on biopsy specimens should be carefully investigated as the current criteria for LCNEC might not always be evaluable in biopsy specimen.

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Chapter 7

An unmet need in the WHO 2015 biopsy classification: Poorly differentiated NSCCs with positive neuroendocrine markers

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To the Editor

It is with great interest that we read the recent World Health Organization (WHO) Classification 2015 of lung tumors and the state-of-the-art concise review by Travis et al. published in the Journal of Thoracic Oncology[1]. The diagnostic criteria for small biopsy specimens are especially interesting and clinically relevant.

The new classification for biopsy specimens to diagnose non–small cell lung carcinoma (NSCLC) without clear morphologic features is driven largely by immunohistochemical (IHC) markers. This classification (figure 7.1) categorizes NSCLC as follows: (1) non–small cell carcinoma (NSCC), favor adenocarcinoma (positive for thyroid transcription factor 1), (2) NSCC, favor squamous cell carcinoma (p63/p40+), or (3) NSCC, not otherwise specified (no morphologic features and negative for IHC markers). Moreover, when neuroendocrine (NE) morphologic features are present, testing for NE markers should be performed, and when the results are positive, the diagnosis "NSCC, favor large cell NE carcinoma (LCNEC)" is preferred. Finally, the diagnostic term for NSCC with morphologic features of NE in the absence of NE IHC markers is NSCC when LCNEC is suspected but stains fail to demonstrate NE differentiation. Following this classification, one category is missing, namely, NSCC without distinct (NE) morphologic features but with positive NE IHC markers, which in this letter is referred as NSCC NE IHC+ (see figure 7.1).

The value of the diagnosis NSCC NE IHC+ has been heavily debated. As many as 10% to 30% of surgically resected NSCLCs have NE differentiation in IHC staining, but no clear association with prognosis has been reported[2,3]. These studies were based on NSCLC with morphologic features of squamous cell carcinoma or adenocarcinoma, however, and often only a single NE IHC marker was positive. Moreover, these studies did not report on poorly differentiated NSCC NE IHC+ in the absence of thyroid transcription factor 1 or P63 staining. Therefore, the value of an NSCC NE IHC+ classification on the basis of biopsy specimens is rather unclear and requires further investigation.

Because an NE growth pattern in biopsy specimens is difficult to recognize, the diagnosis LCNEC may be missed and the incorrect diagnosis of NSCC not otherwise specified made. This misdiagnosis is problematic because the prognosis of LCNEC has been shown to be worse than that of squamous cell carcinomas and adenocarcinomas[4]. Moreover, we have observed that since 2007, the diagnosis of LCNEC on the basis of biopsies has increased dramatically in the Netherlands and that in approximately 54% of cases, the diagnosis was based on positive NE IHC markers and the pathology reports did not mention morphologic features of NE carcinoma[5]. These results indicate that although not advised by the current WHO classification, NE IHC markers have been commonly used in daily practice with undifferentiated carcinomas (NSCCs) in biopsy specimens.

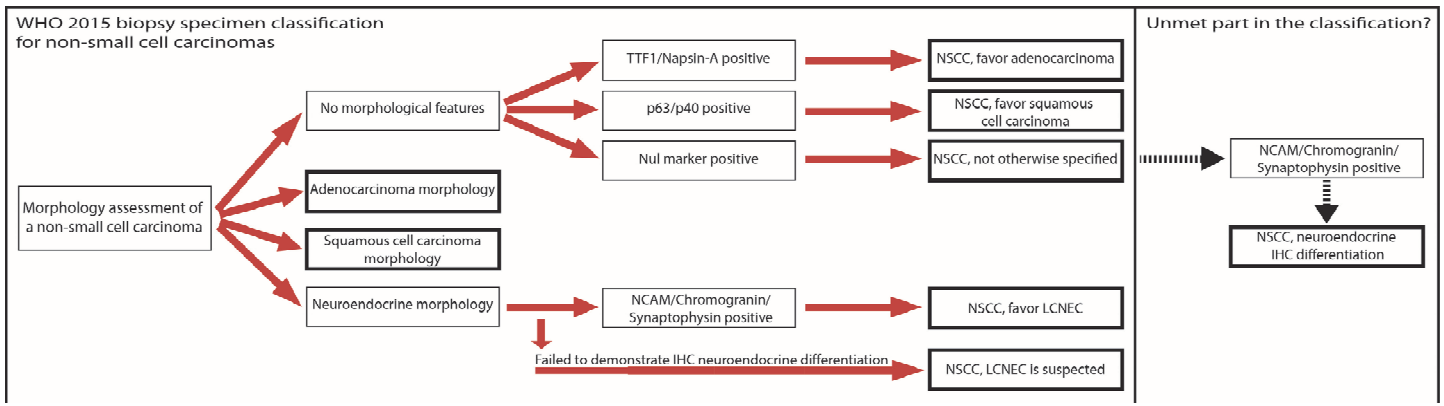

Figure 7.1 An overview of the WHO classification of non–small cell lung carcinomas on biopsy specimens is given. On the right side, the group of NSCCs with positive NE IHC markers, which is not addressed in the 2015 WHO classification, is presented

In conclusion, we think that the diagnosis poorly differentiated NSCC NE IHC+ on the basis of biopsy specimens could be of clinical importance and is an unmet need in the current WHO classification. Future studies should provide more insight into this diagnosis on the basis of biopsy specimens.

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Chapter 8

Searching for diagnostic criteria in pre-operative biopsy specimen for large cell neuroendocrine carcinoma (LCNEC)

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Abstract

Pulmonary large cell neuroendocrine carcinoma (LCNEC) is diagnosed when neuroendocrine morphology and neuroendocrine immunohistochemical staining is present. Recently, arguments were obtained for a different treatment of LCNEC instead of standard NSCLC-NOS therapy. The aim of this study was to establish if new or additional diagnostic features for diagnosing LCNEC on a biopsy specimen could be identified in a cohort consisting of paired LCNEC biopsy samples.

Using the Dutch pathology registry (PALGA) surgically resected LCNEC cases were identified and screened for pre-operative biopsies taken from identical anatomic locations. A blinded review panel systematically screened for all established WHO 2015 criteria on available paired biopsy-resection specimen. Cumulative biopsy sample size was scored.

Between 2003-2012 n=326 patients with surgically resected LCNEC were identified. A biopsy specimen was available in 110 cases and in 60 cases a paired biopsy-resection specimen could be obtained. In 12/60 cases no diagnosis could be established. LCNEC was diagnosed in 32/48 cases on the resection specimen and in 47% (n=15/32) of paired biopsies LCNEC was also confirmed. Neuroendocrine morphology was absent in 53% (n=17/32) of paired biopsies, more often when limited tissue could be evaluated (29% <5 mm (n=14) *versus* 67% ≥5 mm (n=18) *P*=0.04). New insights revealed that ≥2 out of 3 positive neuroendocrine markers may be an argument for the diagnosis of LCNEC in biopsies devoid of neuroendocrine morphology, increasing the sensitivity combined with the established WHO criteria from 47% to 93%.

A minor modification of the current WHO criteria on a biopsy specimen for the diagnosis of LCNEC is proposed. In NSCLC devoid of obvious morphological squamous or adenocarcinoma features, ≥2 out of three positive neuroendocrine IHC stains support a diagnosis of LCNEC. In addition, larger samples or multiple small biopsies may increase the chance to diagnose LCNEC.

Introduction

In lung cancer most diagnosis are made on relatively small samples[1]. Assessing diagnostic histological features pointing to adenocarcinoma, squamous cell carcinoma or LCNEC may be challenging in small biopsies[2]. The distinction between adenocarcinoma and squamous-cell carcinoma in the context of non-small cell lung carcinoma not otherwise specified (NSCLC-NOS) has been addressed in the World Health Organization (WHO) classification of lung tumors of 2015 by the introduction of markers: mucin, TTF1 and P63/P40[3]. More recently, the added value of immunohistochemical (IHC) markers in the differential diagnosis of small-cell lung carcinoma (SCLC) has been described[4].

LCNEC is a high-grade neuroendocrine carcinoma originally described in 1991[5]. The diagnostic criteria for LCNEC according to the WHO classification include frequent mitosis (>10 mitosis/2 mm^2), neuroendocrine morphology such as rosettes, trabecular growth pattern or palisading of cells, and neuroendocrine differentiation identified by IHC markers or electron microscopy. Although LCNEC is made with increasing frequency on small biopsies[6], the diagnostic accuracy and precision for LCNEC to be diagnosed on such specimen is unknown[7,8].

Currently one of the first-line treatment options for non-squamous NSCLC is cisplatinum-pemetrexed chemotherapy[9]. Consequently, metastatic LCNEC tumors that are not recognized as such, because the neuroendocrine morphology is lacking, may be diagnosed as non-squamous NSCLC and treated with cisplatinum-pemetrexed chemotherapy. Recently, inferior outcome in LCNEC of SCLC chemotherapy and cisplatinum-pemetrexed chemotherapy compared to gemcitabine/paclitaxel was reported[10]. Thus, if the diagnosis of LCNEC could be reliably established on small biopsies, a different treatment may be possible than the standard NSCLC-NOS therapy.

The aim of this study was to evaluate in a cohort of LCNEC with paired biopsy-resection specimens if criteria for diagnosing LCNEC on a biopsy specimen could be defined. To this end a nationwide registry was approached and pathology panel review was performed using WHO 2015 criteria in the resection and biopsy specimens.

Material and Methods

Regulations

The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre (METC azM/UM 14-4-043) and was performed according to the Dutch "Federa, Human Tissue and Medical Research: Code of conduct for responsible use (2011)" regulations not requiring patient informed consent.

Patient and tumor selection

In this retrospective population-based study all data were retrieved from the Netherlands Cancer Registry and Netherlands Pathology Registry (PALGA, the nationwide registry of pathology in the Netherlands[11]) as described previously[12]. In short, by screening digital summaries of pathology reports, 994 patients with LCNEC were identified in the combined datasets of patients diagnosed between 01-01-2003 and 31-12-2012. For 326 LCNEC patients the primary tumor was surgically removed. By screening the patient pathology history we identified 110 patients in whom a histopathological biopsy specimen was obtained from the identical anatomic location before surgery (i.e. a paired pre-operative biopsy-resection specimen). Paired biopsy-resection specimen slides were available for 60 of 110 patients (Figure 8.1).

Panel consensus pathology revision

From all histological specimens the original hematoxylin and eosin (H&E) and IHC slides were retrieved. IHC stains included neuroendocrine markers (chromogranin-A, synaptophysin and CD56 (NCAM), TTF1, P63, Ki-67 and available cytokeratin markers all stained in routine diagnosis. All cases minimally included a HE stain and one of three neuroendocrine IHC markers as described previously. Subsequently, three pathologists (RvS, MdB and ET) who were blinded for clinical outcome and for paired biopsy specimens systematically scored all cases at a multi-head microscope. Total tissue size was estimated in the following categories: ≤2, >2 but ≤5, >5 but ≤10 and >10 mm. H&E slides were examined for i) presence of neuroendocrine morphology, ii) estimated mitotic activity in non-crushed fields (≤10, 11-30 or >30 /10 high power field (HPF)), iii) necrosis (none, 'dot-like' [=as occasionally seen in atypical carcinoids] or abundant [=more extensive than 'dot-like']). The MIB1 (Ki-67) staining was scored (<25%, >25%) when available[13]. In more limited tissue samples (<2 mm^2), mitosis were evaluated on all assessable HPF's[4]. Either >10 mitosis/2mm^2, abundant tumor necrosis, or a Ki-67 staining of more than 25% of tumor cells was sufficient to score for high-grade tumor disease[13]. Chromogranin-A and synaptophysin were scored as (+) on observation of focal small

cytoplasmic dots in an occasional tumor cell at 40x microscope. Any membrane staining was sufficient for CD56 (+). For all neuroendocrine markers observation of staining (4x or 2.5x objective) was scored as strongly positive (+++), and in between staining as (++). Additionally, p63/p40/TTF1 and cytokeratin staining were evaluated when available. Diagnoses were established according to the algorithm as described in the WHO classification (2015).

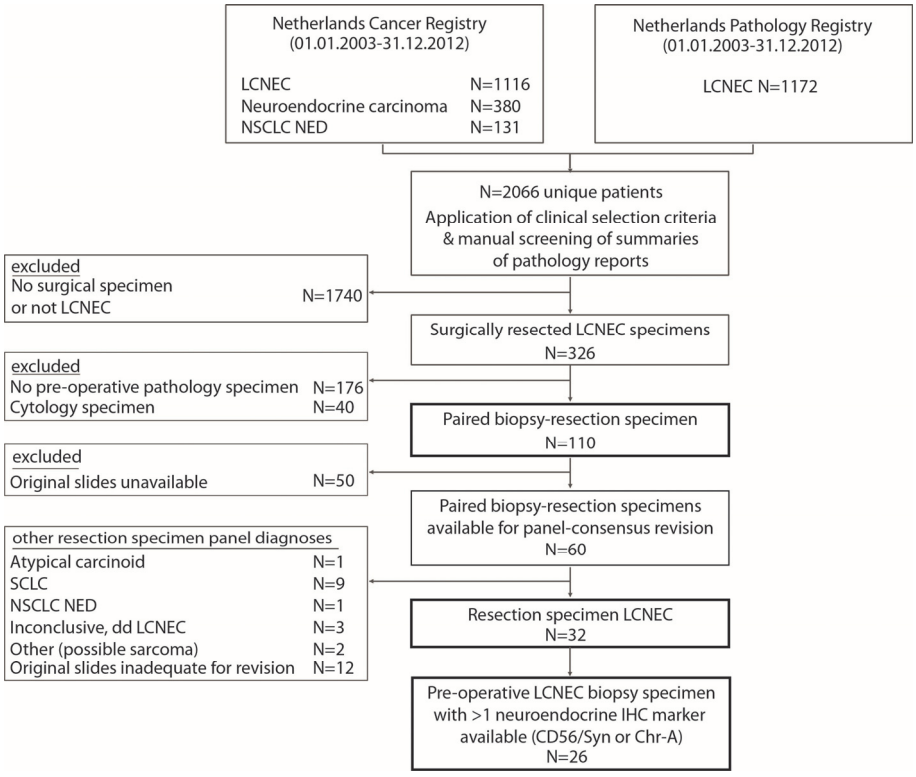


Figure 8.1 Selection of surgical LCNEC specimen with pre-operative biopsy specimen available for panel review
Abbreviations: N, number; LCNEC, large cell neuroendocrine carcinoma; NSCLC NED, non-small cell lung carcinoma with immunohistochemical neuroendocrine differentiation; SCLC, small cell lung carcinoma; IHC, immunohistochemistry; Syn, synaptophysin; Chr-A, chromogranin-A

Statistics

All analyses were performed using SPSS (version 22 for Windows, Inc., Chicago, IL). To compare categorical data χ^2 and Fisher Exact test were used. Two-sided *P*-values <0.05 were considered significant.

Results

In a series of 110 LCNEC diagnosed on the resected specimen (Figure 8.1), the original diagnoses of the paired pre-operative biopsy specimen included, LCNEC (22%, n=24), NSCLC (42%, n=47), SCLC (16%, n=18), high-grade neuroendocrine carcinoma (6%, n=6), carcinoid (6%, n=6), and other (8%, n=9) illustrated in Figure 8.2A and diagnostic details can be found in Table S8.1.

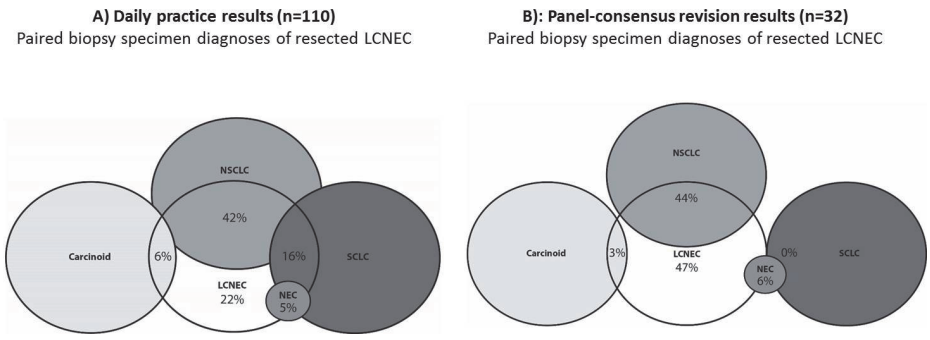


Figure 8.2 **A**) Overview of diagnoses established on a paired pre-operative biopsy vs. resection specimen in daily practice (n=110) and **B**) by panel-consensus revision (n=32), samples were taken from identical anatomic regions. The missing 9% of figure A can be found in Table S8.1.
Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung cancer; NEC, neuro-endocrine carcinoma.

Sixty out of these 110 samples were obtained for our central panel review leading to the following results: first, on the resection specimens a definitive diagnosis was not possible to be established on 12 cases because of inadequate original tumor slides. Of the remaining 48 resection specimen the diagnosis was LCNEC n=32, SCLC n=9, atypical carcinoid n=1, NSCLC n=2, possible sarcomas n=2, and no conclusive diagnosis n=2. Second, on the paired pre-operative biopsy specimen in only 15 of the 32 (47%) the diagnosis of LCNEC could be established. Other diagnoses included NSCLC (44%), carcinoid (3%) and inconclusive (6%) (Figure 8.2B).
An overview of all diagnostic criteria identified in the paired pre-operative biopsy specimens with a final diagnosis of LCNEC on the resection specimen is presented in Table 8.1A; specimens with a resection diagnosis different than LCNEC are presented in Table 8.1B.

Table 8.1A Results of structured WHO criteria evaluation in biopsy specimen from surgical resection specimen diagnosed as LCNEC

Resection	Biopsy	Specimen		Grading				Morph.	Neuroendocrine IHC				Other IHC	
		Type	*Size (mm)*	*Mit.*	*Nec.*	*Ki-67%*	*High-grade*	*Neuro.*	*CD56*	*Syn*	*Chr-A*	*≥2+*	*TTF1*	*P63*
LCNEC	LCNEC	Needle	>5-10	>10	+/-	>25	+	+	UA.	++	-	No	+	UA.
LCNEC	LCNEC	Needle	>10	>10	+	UA.	+	+	++	-	-	No	-	UA.
LCNEC	LCNEC	Needle	>10	>30	+	UA.	+	+	+	UA.	UA.	UA.	-	UA.
LCNEC	LCNEC	EBB/TBB	>2-5	>30	+	UA.	+	+	+++	UA.	UA.	UA.	-	-
LCNEC	LCNEC	Needle	>10	>10	+	UA.	+	+	+	++	++	Yes*	-	-
LCNEC	LCNEC	Needle	>10	>10	+	UA.	+	+	+++	++	-	Yes*	+	UA.
LCNEC	LCNEC	Needle	>5-10	>10	+	>25	+	+	++	++	++	Yes	-	UA.
LCNEC	LCNEC	Needle	>10	>10	+	UA.	+	+	-	+	++	Yes	UA.	UA.
LCNEC	LCNEC	EBB/TBB	<2	>10	-	>25	+	+	++	UA.	+	Yes	+	-
LCNEC	LCNEC	Large BB	>10	>30	+	UA.	+	+	++	+++	-	Yes	+	UA.
LCNEC	LCNEC	Needle	>10	>30	+	UA.	+	+	-	+	++	Yes	-	-
LCNEC	LCNEC	EBB/TBB	>2-5	>10	+	UA.	+	+	+	+++	-	Yes	+	UA.
SCLC-LCNEC	LCNEC	Needle	>10	>30	-	>25	+	+	+++	UA.	++	Yes*	+	UA.
NSCLC-LCNEC	LCNEC	EBB/TBB	<2	>30	-	>25	+	+	++	+	+	Yes	-	UA.
SqCC-LCNEC	SqCC-LCNEC	Needle	>10	>10	+	>25	+	+	++/-	-	-	No	+	-/+
LCNEC***	AC	EBB/TBB	<2	<10	-	<25	-	+	+	UA.	+++	Yes	-	UA.
LCNEC	Favor AdC	EBB/TBB	<2	UA.	-	UA.	-	-	++	++	++	Yes	+	UA.
LCNEC	Favor AdC	Needle	>10	>30	+	>25	+	-	+	+	-	Yes	+	-
LCNEC	NSCLC NOS	Needle	>2-5	>30	+	>25	+	-	-	+++	++	Yes	-	-
LCNEC	Favor AdC	EBB/TBB	>5-10	>30	+	>25	+	-	-	+	-	No	+	UA.
LCNEC	Favor AdC	EBB/TBB	<2	UA.	-	>25	+	-	+	UA.	UA.	UA.	+	-
LCNEC	Favor AdC	EBB/TBB	>2-5	UA.	-	<25	-	-	+	UA.	++	Yes	+	-
LCNEC	Favor AdC	Needle	>5-10	<10	+	UA.	-	-	-	++	+++	Yes	+	UA.
LCNEC	NSCLC NOS	EBB/TBB	<2	>10	-	UA.	+	-	+	UA.	++	Yes	-	-
LCNEC	NSCLC NOS	EBB/TBB	>2-5	<10	-	UA.	-	-	++	-	++	Yes	-	UA.
LCNEC	NSCLC NOS	EBB/TBB	>2-5	>10	+	UA.	+	-	++	++	-	Yes*	UA.	UA.
LCNEC	NSCLC NOS	Needle	>10	<10	+	>25	+	-	++	+	-	Yes	-	UA.
LCNEC	NSCLC NEM	Needle	>10	>10	+	>25	+	+	-	-	-	No	-	Focal
LCNEC	Favor AdC	EBB/TBB	<2	>10	-	UA.	+	-	UA.	UA.	-	UA.	+	UA.
SqCC-LCNEC	SqCC	EBB/TBB	>10	UA.	-	UA.	-	SqCC	UA.	UA.	UA.	UA.	UA.	UA.
LCNEC	NSCLC vs SCLC	EBB/TBB	<2	UA.	+	-	+	-	+++	UA.	UA.	UA.	UA.	UA.
LCNEC	NSCLC vs SCLC	Needle	>10	>30	+	-	+	-	++	+	-	Yes	-	-

Table 8.1B Results of structured WHO criteria evaluation in biopsy specimen from surgical resection specimen not diagnosed as LCNEC

Resection	Biopsy	Specimen		Grading				Morph.	Neuroendocrine IHC				Other IHC	
		Type	*Size*	*Mit.*	*Nec.*	*Ki-67*	*High-grade*	*Neuro.*	*CD56*	*Syn*	*Chr-A*	*≥2+*	*TTF1*	*P63*
SCLC	LCNEC	Needle	>5-10	>10	+	>25	+	+	++	UA.	UA.	UA.	+	-
SCLC	LCNEC	Needle	>10	<10	+	UA.	-	+	++	-	+	Yes	-	UA.
SCLC	Favor AdC	Needle	>10	>30	-	UA.	+	-	++	-	+	Yes	+	Foc.
SCLC	Favor AdC	Needle	>10	>30	+	>25	+	-	+++	UA.	UA.	UA.	+	UA.
SCLC	NSCLC NOS	EBB/TBB	>2-5	>10	+/-	UA.	+	-	+++	UA.	++	Yes	UA.	UA.
SCLC	NSCLC NOS	Needle	>2-5	UA.	+	UA.	-	UA.	++	-	-	No	-	UA.
SCLC	NSCLC NOS	Needle	>10	UA.	+	UA.	-	UA.	+	UA.	++	Yes	-	UA.
SCLC	NSCLC vs. SCLC	EBB/TBB	>2-5	<10	-	UA.	-	-	+	+	-	Yes	UA.	-
SCLC	SCLC	EBB/TBB	<2	UA.	-	UA.	-	UA.	+	+	+	Yes	+	UA.
NSCLC NED vs.LCNEC vs SCLC	Favor AdC	Needle	>10	>10	+	UA.	+	-	-	++	++	Yes	+	-
NSCLC NED vs LCNEC vs SCLC	Favor AdC	Needle	>10	>10	+	>25	+	-	+++	++	+	Yes	+	-
AC (borderline)	AC	EBB/TBB	>10	<10	-	<10	-	+	++	+++	+++	Yes	-	-
LCNEC vs SCLC vs AC	LCNEC	EBB/TBB	>2-5	>10	-	>25	+	+	++	-	++	Yes	+	UA.
NSCLC NED	NSCLC NOS	Needle	>10	>30	+	UA.	+	-	+	-	-	No****	-	-
Carcino sarcoma	NSCLC NEM	EBB/TBB	>5-10	>30	-	UA.	+	+	-	-	-	No	UA.	UA.
DD sarcoma	Favor AdC	EBB/TBB	<2	UA.	-	UA.	-	UA.	+	UA.	UA.	UA.	+	UA.

* IHC staining is performed only on the biopsy specimen. ** Although no neuroendocrine morphology was observed in these small biopsies panel consensus was LCNEC as neuroendocrine necrosis and cell-type were observed and multiple IHC markers stained positive. *** Resection sample with borderline LCNEC based on mitosis but importantly no abundant necrosis was observed, in Ki-67 strong heterogeneity was observed (<25% and >25%). .**** IHC staining is performed only on the resection specimen or the biopsy specimen.† Favored LCNEC diagnoses although mitosis and necrosis could not be observed, Ki-67 would have been preferred but was not available. †† Neuroendocrine part is positive for CD56 but negative for P40. ††† Samples without necrosis or sufficient mitosis as only small fields could be observed. However, cases were diagnosed as LCNEC on the resection specimen

Abbreviations: UA, unavailable; IHC, immunohistochemistry; NOS, not otherwise specified; NSCLC NED, NSCLC with neuroendocrine IHC differentiation; AdC, adenocarcinoma; SqCC, squamous cell carcinoma; AC, atypical carcinoid; EBB, endobronchial biopsy; TBB, trans bronchial biopsy; Syn, synaptophysin; Chr-A, chromogranin-A; Foc. Focal; SCLC, small cell lung carcinoma; LCNEC, large cell neuroendocrine carcinoma; dd, differential diagnosis; NEM; neuroendocrine morphology without staining for neuroendocrine markers; vs, versus

WHO criteria for LCNEC diagnosis were to a variable extend observed in the biopsy specimen. In 50% of the cases neuroendocrine morphology was absent (Table 8.2). In biopsies with cumulative size of >5 mm tumor tissue the characteristic 'neuro-endocrine morphology" was more frequently present compared to smaller samples in 67% and 29%, respectively (*P*=0.04). Cumulative size revealed no differences for the presence of mitosis, nucleoli, cytoplasm and necrosis (Table 8.2). Examples are shown for i) neuroendocrine morphology ii) vague neuroendocrine morphology or iii) absent neuroendocrine morphology in the pre-operative biopsy specimen but confirmed in the LCNEC resection specimen is presented in Figure 8.3A-F.

For 26 out of 32 cases with a resection confirmed diagnosis of LCNEC ≥2 neuroendocrine markers including chromogranin-A, synaptophysin or CD56 could be retrieved (Figure 8.1). In 80% (21/26) of these cases positive staining for at least two neuroendocrine markers was observed. In 11/15 (73%) biopsies with neuroendocrine morphology staining for ≥2 neuroendocrine markers was observed. In 11 paired cases, where resection specimen was classified as LCNEC, but without neuroendocrine morphology in the biopsy specimen, ≥2 neuroendocrine IHC markers were available. In 10/11 (91%) of these cases ≥2 neuroendocrine IHC markers were positive. If in addition to neuroendocrine morphology the presence of ≥2 neuroendocrine IHC markers was considered a marker for LCNEC in the context of an undifferentiated carcinoma, then the diagnosis of LCNEC increased from 47% (n=15/32) to 78% (n=25/32) on pre-operative biopsy specimens (exemplary case Figure 8.3E-F, Table 8.1A). Biopsy cases staining for ≥2 neuroendocrine markers and combined with neuroendocrine morphology corresponded to a LCNEC diagnosis of 93% (n=26/28) on resection

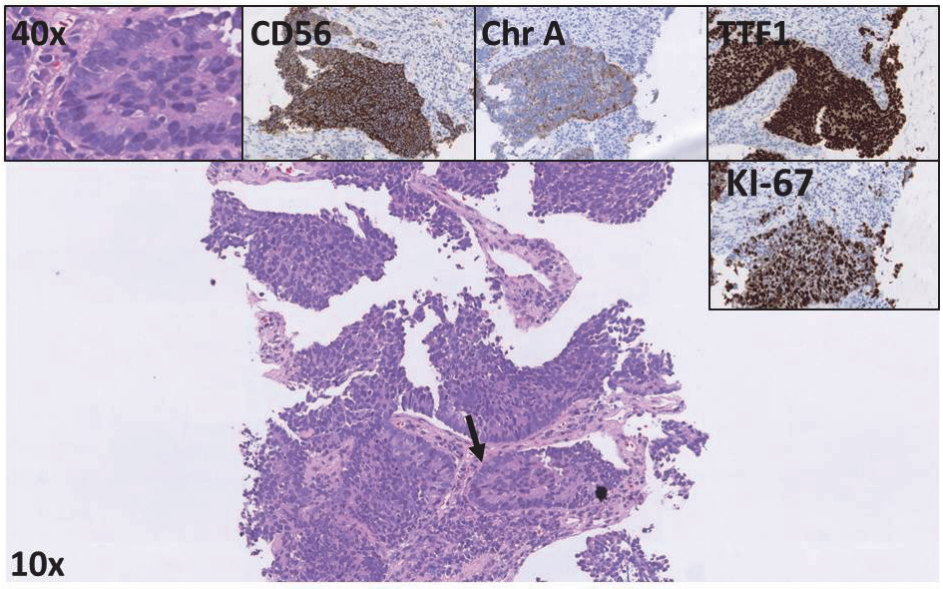
In the panel reviewed cohort 9 SCLC diagnoses were established on the resection specimen. Two of these cases were diagnosed as LCNEC pre-operatively, one as SCLC, one had a differential diagnosis of SCLC *versus* NSCLC*,* and 5 were diagnosed as NSCLC. In 7 cases ≥2 neuroendocrine markers were available and 6/7 (86%) stained positive for ≥2. The remaining three cases were classified as not neuroendocrine in the resection specimen, 2/3 had neuroendocrine markers available and 0/2 stained for ≥2 neuro-endocrine markers.

Table 8.2 Comparison of WHO criteria evaluated in the paired pre-operative biopsy and resection specimens of LCNEC

	Specimen type				p-value*
	Pre-operative biopsy			Resection	<5mm vs. >5mm
WHO 2015 criteria	*Total*	*<5mm*	*≥5mm*	*Total*	
Total (n)	32	14	18	32	-
Mitosis (% scored as in resection specimen)	72%	57%	83%	-	0.13
≤10	4	2	2	0	
>10	23	8	15	31	
Not assessable	5	4	1	1	
Necrosis (% scored as in resection specimen)	63%	50%	72%	-	0.28
Large zones	20	5	15	26	
Dotlike (focal necrosis as in AC)	1	0	1	4	
No necrosis	10	9	1	2	
Not assessable	1	0	1	0	
Neuroendocrine morphology (% scored as in resection specimen)	50%	29%	67%	-	0.04**
Not present	9	4	5	0	
Present	15	5	10	31	
Heterogeneous among pathologists	3	0	3	1	
Not assessable	5	5	0	0	
≥2/10 large (non-inconspicuous) nucleoli (% scored as in resection specimen)	59%	50%	67%	-	0.47
No	28	11	17	18	
Yes	3	3	0	13	
Heterogeneous among pathologists	0	0	0	1	
Not assessable	1	0	1	0	
Cytoplasm as in NSCLC (% scored as in resection specimen)	88%	93%	83%	-	0.61
No	1	1	0	1	
Yes	29	13	16	30	
Heterogeneous among pathologists	1	0	1	1	
Changing within specimen (i.e. yes and no)	0	0	0	0	
Not assessable	1	0	1	0	
Molding (% scored as in resection specimen)	69%	86%	55%	-	0.12
No	25	13	12	25	
Yes	3	0	3	0	
Heterogeneous among pathologists	2	0	2	6	
Not assessable	2	1	1	1	

* Fisher Exact test comparing number of paired and resection specimens identically scored for WHO criteria subcategory. ** Chi-square test comparing number of paired and resection specimens identically scored for WHO criteria subcategory (i.e. neuroendocrine morphology present in both specimens)
Abbreviations: NSCLC, non-small cell lung carcinoma, AC, atypical carcinoid; mm, millimeters

A



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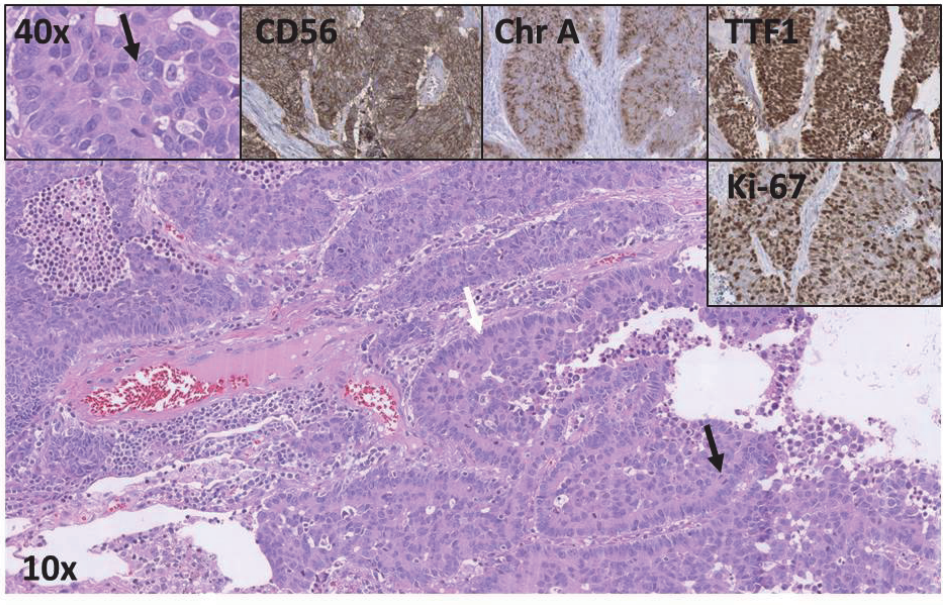

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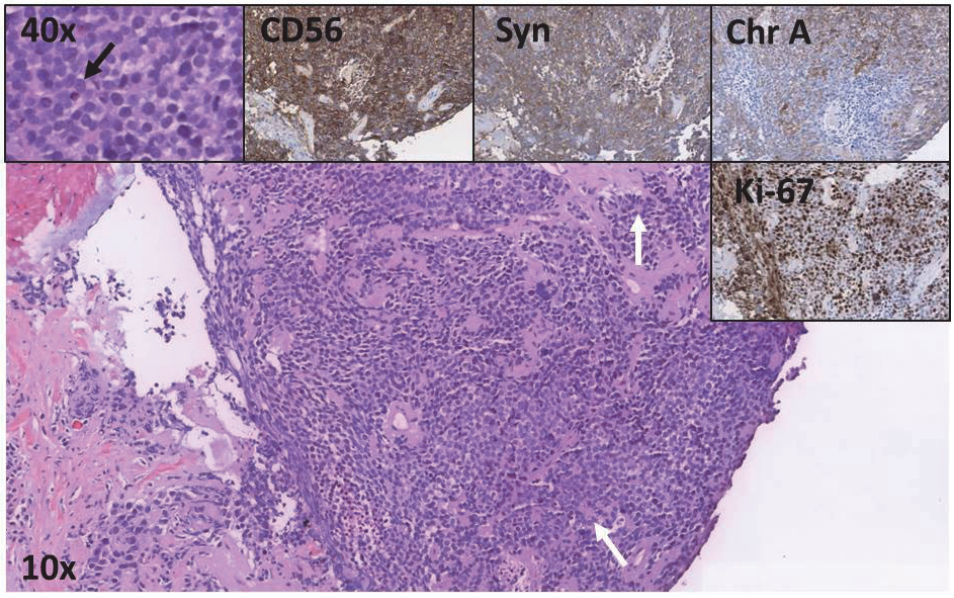

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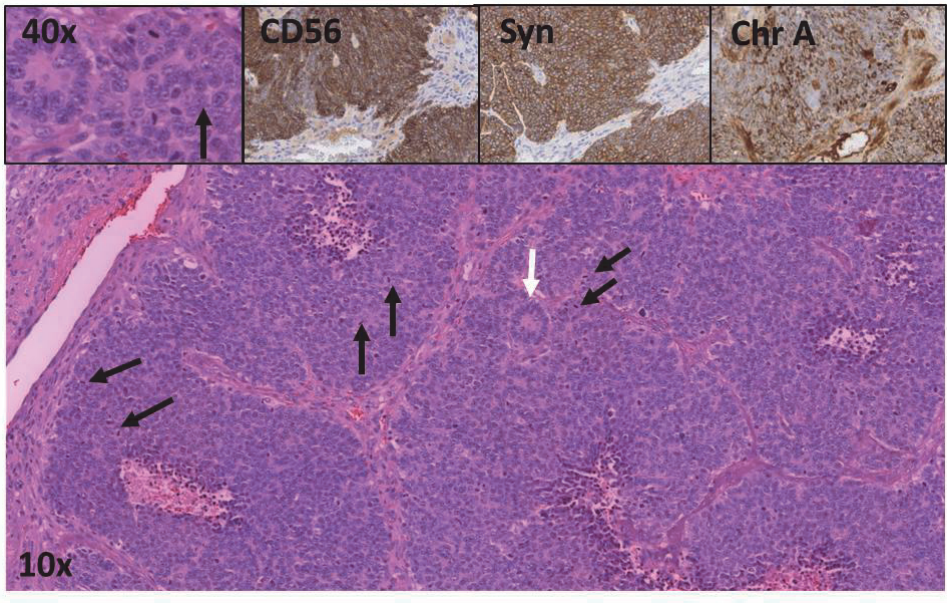

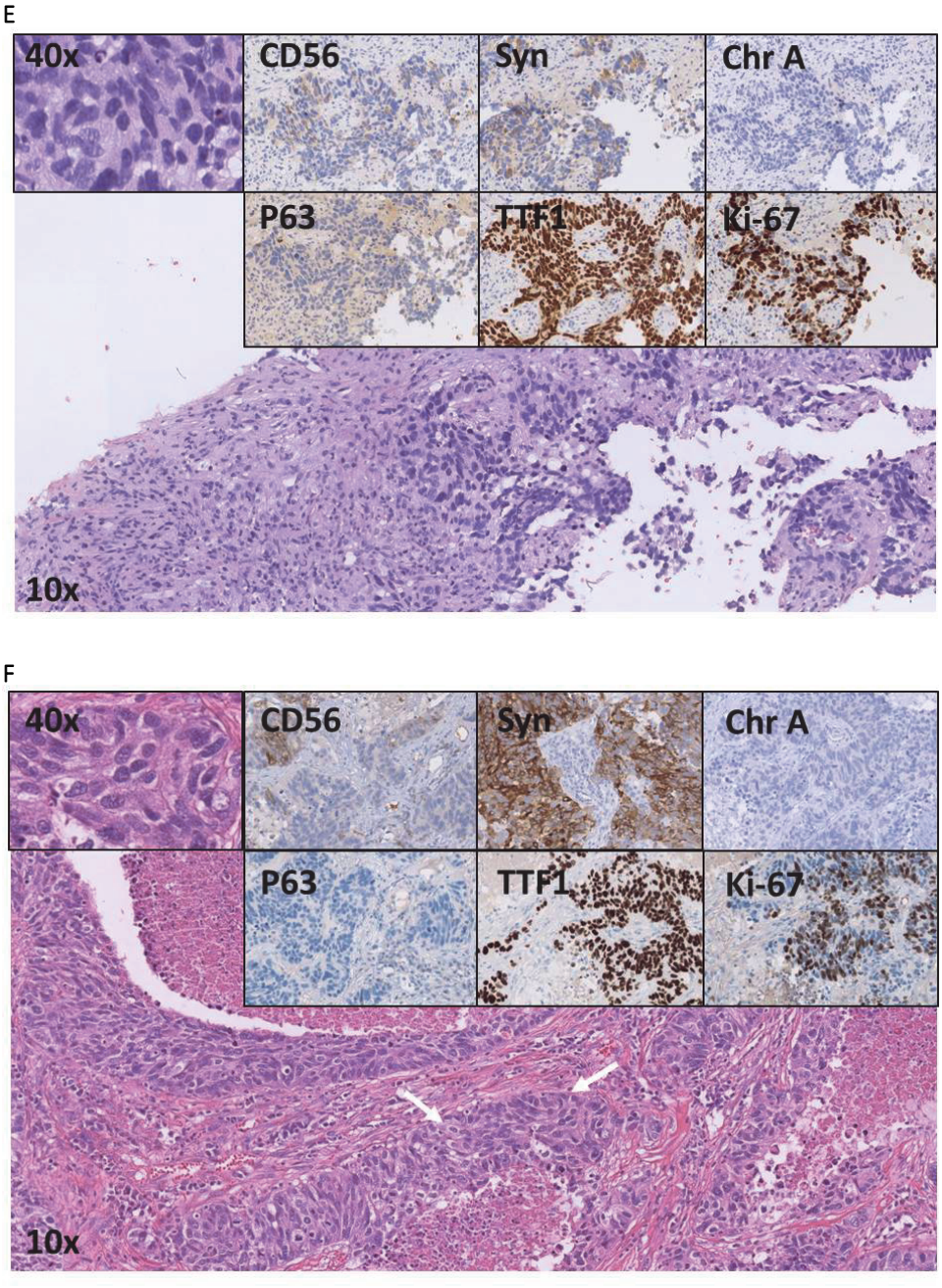

Figure 8.3 **A-F**) Overview of paired pre-operative biopsy-resection specimens' consensus diagnosed as LCNEC on the resection specimens.
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma.

Biopsy specimen A) (matched with resection specimen B):
According to the established WHO classification, **NSCLC favor LCNEC** would be diagnosed.
Neuro-endocrine morphology (palisading, arrow) is observed. The left upper panel (40x) shows cells with non-small cell cytological features. CD56 and Chromogranin-A staining confirm neuroendocrine differentiation (upper middle panels), TTF1 is positive (upper right) while high-grade disease is confirmed with the Ki-67 staining (>25%, lower right panel).

Resection specimen B) (matched with biopsy specimen A):
According to the established WHO classification, **LCNEC** would be diagnosed.
Identical to the biopsy specimen, neuro-endocrine morphology is present (palisading, white arrow). In the left upper panel (40x) non-small cell cytological features can be observed with abundant cytoplasm and nucleoli (arrow). The black arrow (10x overview) highlights a mitosis while the Ki-67 (lower right panel) confirms high-grade disease (>25%).

Biopsy specimen C) (matched with resection specimen D):
According to the established WHO classification, **NSCLC favor adenocarcinoma** would be diagnosed.
On the overview (10x) an undifferentiated tumor is seen with few rosettes (white arrows) cytological features of a non-small cell with modest cytoplasm and nucleoli (arrow, 40x left upper panel). TTF1 and P63 were not available. The neuroendocrine marker CD56 showed strong membranous staining, synaptophysin and chromogranin-A showed modest to strong cytoplasmic staining (granular, right upper panels). Ki-67 was high (>25%).

Resection specimen D) (matched with biopsy specimen C):
According to the established WHO classification, LCNEC would be diagnosed.
On the overview (10x) neuroendocrine morphology is observed (rosette, white arrow) cytological features of a non-small cell with abundant cytoplasm and nucleoli (arrow, 40x left upper panel). Multiple mitosis are observed (black arrows). Neuroendocrine marker staining is similar to the biopsy specimen.

Biopsy specimen E) (matched with resection specimen F):
According to the established WHO classification, **NSCLC favor adenocarcinoma** would be diagnosed.
Note: according to the current study the proposed diagnosis will be **LCNEC**, confirmed in the resection specimen (F).
On the overview (10x) an undifferentiated NSCLC is observed (cytological features 40x, left upper panel). P63 is negative but TTF1 is strongly positive (middle lower panel). High-grade disease is confirmed with the Ki-67 (>25%, lower right panel). The neuroendocrine marker CD56 shows modest membranous staining (upper middle left), synaptophysin shows granular staining (upper middle right) while chromogranin-A is negative (upper right).

Resection specimen F) (matched with biopsy specimen E):
According to the established WHO classification, LCNEC would be diagnosed.
On the overview (10x) a neuroendocrine morphology is present (white arrows) and cytological features of a non-small cell with abundant cytoplasm (40x, left upper panel). Immunohistochemical markers show identical patterns as the biopsy specimen (middle lower and upper panels).

Discussion

Our retrospective study shows that in small biopsy samples the presence of ≥2 out of three positive neuroendocrine marker supports a diagnosis of LCNEC in cases devoid of neuroendocrine morphology (i.e. undifferentiated NSCLC). The sensitivity of the

diagnosis LCNEC may thus be increased to 93%, providing a chance for better treatment in patients with LCNEC.

Staining for at least two IHC neuroendocrine markers (such as chromogranin-A, synaptophysin or CD56) is a characteristic feature that has been reported in LCNEC but which is absent in virtually all NSCLC. NSCLC may show focal IHC staining for one neuroendocrine marker in 8-31% of squamous cell carcinoma and 17-33% of adenocarcinoma (Table 8.3)[14-20]. Importantly, positive staining of ≥2 out of 3 neuroendocrine markers is reported in less than 1% of NSCLC and triple positive neuroendocrine differentiation has not been reported in NSCLC[19]. In contrast, positive IHC for ≥2 out of 3 neuroendocrine markers is observed in ≥80% of LCNEC, and triple positive in ≥60%[21,22]. Hence, application of the criterion for ≥2 out of 3 neuroendocrine markers positive neuroendocrine marker staining has a high sensitivity for a diagnosis of LCNEC on a biopsy based on our study and a high specificity based on the abovementioned literature, providing a rational to diagnose LCNEC in the context of undifferentiated NSCLC with neuroendocrine phenotype as likely being LCNEC.

In our panel review series and in the originally established diagnoses, LCNEC was frequently diagnosed as undifferentiated NSCLC with a preferred diagnosis off adenocarcinoma when TTF1 was positive on the pre-operative biopsy specimen. TTF1 shows staining in 23-83% of LCNEC[23-26]; P40 and P63 are used to diagnose 'NSCLC favor squamous cell carcinoma, are almost always negative in LCNEC but can show focal staining[25,26]. Applying the criterion of staining of ≥2 out of 3 neuroendocrine markers may resolve the underrecognition of LCNEC in cases devoid of neuroendocrine morphology. In addition to LCNEC, this criterion was also applicable for the SCLC cases identified in this panel review study.

The value of neuroendocrine immunohistochemical staining (i.e. differentiation) in NSCLC has been investigated in the past but was largely focused on resection specimen[14-16,18-20]. Based on these studies the established WHO classification advises that neuroendocrine markers should not be performed when neuroendocrine morphology is not observed[3]. The main argument for this is that these neuroendocrine markers are in NSCLC not of prognostic or therapeutic relevance and therefore should, according to the WHO, not been performed[27]. The small adjustment proposed in this study is provided in a diagnostic flowchart in Figure 8.4.

Table 8.3 Overview of neuroendocrine IHC marker staining in adenocarcinomas and squamous cell carcinomas and reported clinical relevance

Authors	Journal	Year	Stage	TMA	Morphology	Any [+]	CD56	Chr-A	Syn	Conclusion
González Argoneses et al.	Cancer	2007	I	Yes (>10% of cells)	AdC N=156 SqCC N=162	-	-	-	52/156 (33%) 34/128 (21%)	Worse prognosis for NSCLC NED
Pelosi et al.	Cancer	2003	I	No (Median of 2000 cells)	AdC N=88 SqCC N=113	15 (17%) 13 (12%)	-	0.0% 1.57%	4.0% 5.8%	>5% positivity for Chr-A/Syn relates with poor prognosis
Segawa et al.	J Cancer Res Clin Oncol	2009	UA.	No (Focal or more)	AdC N=55 SqCC N=50	15 (27% 4 (8%)	7 (18%) 2 (4)	4 (7) 1 (2)	8 (15) 0 (0)	NSCLC NED has no clear relation with prognosis
Sterlacci et al.	Vichows Arch	2009	I-IV	Yes (any positivity)	AdC N=197 SqCC N=119	38 (19%) 7 (6%)	10 (5%) 3 (3%)	2 (1%) 0 (0%)	28 (16%) 4 (3%)	NSCLC NED has no clinical significance
Hage et al.	Chest	1998	I-III	No (UA.)	AdC N=262 SqCC N=575	-	30 (11) 86 (15%)	-	-	NSCLC NED has no relation with prognosis
Ionescu et al.	Am J Surg Pathol	2007	UA.	Yes (>1% cell positivity)	AdC N=243** SqCC N=272**	76 (31%) 83 (31%)	11 (5.1%) 29 (12.4%)	1 (0.4%) 1 (0.4%)	23 (11.2%) 10 (4.3%)	NSCLC NED has no clinical significance**
Howe et al.	Histopathology	2005	I-III (A) >III (B)	Yes (A), No (B) (> focal weak)	NSCLC* (A) N=341 NSCLC* (B) N=98	157 (36%)	A) 27,5% B) 29%	A) 6,2% B) 1%	A) 16,5% B) 17,6%	NSCLC NED has no relation with prognosis or chemotherapy response

* Combination of morphological differentiated AdC, SqCC, large cell carcinoma and adeno squamous carcinomas. ** Additionally analyzed for double staining: Syn[+] & Chr-A[+] AdC/SqCC n=1/0, Syn[+] & CD56[+] AdC/SqCC n=2/2, Chr-A[+] & CD56[+] AdC/SqCC n=0/0, 3 marker[+] AdC/SqCC n=0/0

Abbreviations: AdC, adenocarcinoma; SqCC, squamous cell carcinoma; Syn, synaptophysin, Chr-A, chromogranin-A; UA; unavailable; TMA, tissue microarray

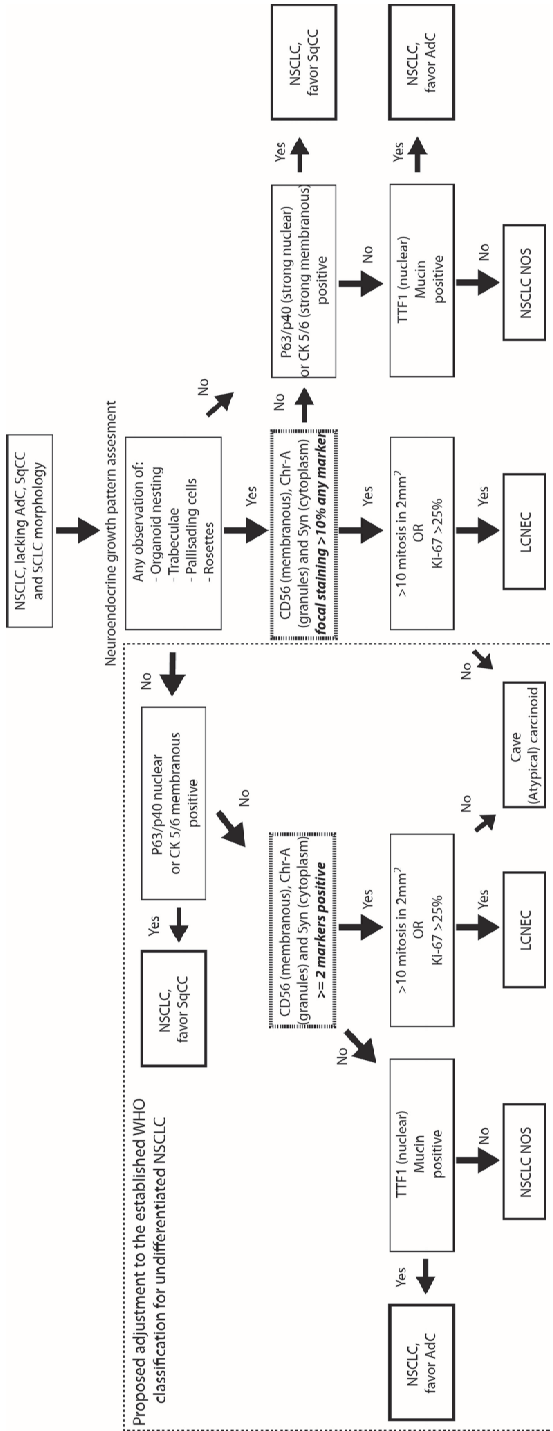

Figure 8.4 Suggested adjustments to the established World Health Organization (WHO) 2015 classification diagnostic algorithm for non-small cell lung cancer on biopsy specimen.
Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; NSCLC, non-small cell lung cancer; AdC, adenocarcinoma; SqCC, squamous cell carcinoma; NOS, not otherwise specified; Chr-A, chromogranin-A; SCLC, small cell lung cancer

One previous study compared pre-operative biopsy specimen taken from identical anatomic location of surgically confirmed LCNEC. In all 6 cases the diagnosis of LCNEC was made on biopsy specimen. However, cumulative biopsy size was not provided[7]. Our study clearly indicates that the tendency to diagnose LCNEC on a biopsy specimen is low and therefore in clinical practice LCNEC tumors are commonly diagnosed as NSCLC. This observation is further strengthened by the performance of a blinded pathology review showing similar results.

A recent study demonstrated that LCNEC is an umbrella term, encompassing cases overlapping with SCLC, NSCLC and carcinoids[4]. Considerable inter-observer variation between and some biological similarities between LCNEC and SCLC contribute in this aspect[4,28,29]. Morphometric studies identified that the cytological criteria, used to separate LCNEC from SCLC, show significant overlap mainly in cell size[30]. Here we observed that several NSCLCs diagnosed on biopsy were diagnosed as SCLC on the resection specimen. Cytological features may be different in larger tissue samples, possibly explaining some of the overlap observed between these cases[31]. Finally, tumor heterogeneity may be an issue, biopsy specimen taken from the LCNEC component of a combined SCLC-LCNEC was also observed in our cohort[31]. Absence of P16 staining and presence of RB1 staining may favor a diagnosis of LCNEC (and NSCLC)[4].

Diagnostic overlap between high-grade neuroendocrine carcinomas and carcinoid, especially in crushed biopsy samples, has been described previously[32]. In our evaluations we only found a limited overlap in routine practice (6%, n=6) and by panel review (3% n=1, Figure 8.2A & B). Nevertheless, mitosis and necrosis were commonly difficult to evaluate in LCNEC biopsy specimen as also indicated by others[33]. To increase the diagnostic discrimination of LCNEC from carcinoid in biopsy specimen, the use of proliferation markers such as Ki-67 has been suggested[32]. Thus far, Ki-67 evaluation by so-called “eyeballing” seems to be the most pragmatic approach in limited tissue in daily practice with a cut-off at ≥25% to define high-grade neuroendocrine carcinoma[33,34].

Overlap with undifferentiated NSCLC on biopsy specimens mainly occurred because of an absence of neuroendocrine morphology. Our study provided an argument that the difficulty a pathologist may perceive in diagnosing LCNEC in small specimens mainly results from the small cumulative sample size, prohibiting adequate identification of neuroendocrine morphology. Larger and more biopsies will likely facilitate LCNEC diagnosis.

Our study was limited by the limited number of paired biopsy-resection specimens analyzed due to the unavailability of slides was available for panel review. Nevertheless, the described diagnostic issues in this study reflect the daily clinical practice closely. Furthermore, this is the first substantial analysis of the established WHO criteria for LCNEC on biopsy specimen using matched resection specimen as golden standard. A further limitation is that not in all cases >1 neuroendocrine stain was available. Both issues hamper the power of the study. Finally, this retrospective study should be validated in another cohort.

In conclusion, we propose a minor modification of the current WHO criteria for biopsy specimens (Figure 8.4). An undifferentiated carcinoma may be classified as likely LCNEC on a biopsy specimen when the following features are present: i) non-small cell cytological features, ii) at least two out of three positive neuroendocrine (chromogranin A, synaptophysin, CD56) stains and iii) high-grade proliferative activity (by evaluation of mitosis or Ki-67). Furthermore, pathologists are encouraged to request clinicians to sample tumors with larger core biopsies or take multiple small biopsies to increase the amount of tissue evaluable.

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Supplemental table

Table S8.1 LCNEC diagnoses established on a pulmonary resection specimen

Variable	Pulmonary Resection specimen *n (%)*	Pre-operative biopsy specimen *n (%)*
Resected specimen diagnosis		
LCNEC*	277 (85)	94 (87)
Combined LCNEC-SqCC	18 (5)	6 (5)
Combined LCNEC-AdC	17 (5)	6 (5)
Combined LCNEC (NOS)	9 (3)	2 (2)
NSCLC, favor/possible LCNEC	5 (2)	1 (1)
Pre-operative sampling method		
Needle biopsy	25 (17)	25 (23)
EBB/TBB or NOS	85 (56)	85 (77)
Cytology	40 (27)	-
Paired pre-operative biopsy specimen diagnosis		
LCNEC*	25 (16)	24 (22)
SCLC	25 (16)	18 (16)
NSCLC	72(47)	47 (42)
High-grade neuroendocrine carcinoma**	7 (5)	6 (6)
Carcinoid (atypical, typical or NOS)	7 (5)	6 (6)
Neuroendocrine tumor NOS	1 (1)	1 (1)
Carcinoid or high-grade neuroendocrine carcinoma	1 (1)	1 (1)
NSCLC NED or LCNEC	1 (1)	1 (1)
No conclusive diagnosis	4 (3)	3 (3)
Other	8 (5)	3 (3)

* Including the diagnosis NSCLC neuroendocrine carcinoma, combined LCNEC, NSCLC favor/possible LCNEC.
** Including the diagnoses: “high grade neuroendocrine carcinoma”, “neuroendocrine carcinoma of intermediate cell type”, “high grade neuroendocrine carcinoma with differential diagnosis of LCNEC vs. SCLC”.
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; SqCC, squamous cell carcinoma; AdC, adenocarcinomas; NOS, not otherwise specified; N, number; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; NED, neuroendocrine differentiation; EBB, endobronchial biopsy; TBB, trans bronchial biopsy

Chapter 9

Chemotherapy for pulmonary large cell neuroendocrine carcinomas: does the regimen matter?

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* Authors contributed equally

Abstract

Pulmonary large cell neuroendocrine carcinoma (LCNEC) is rare. Chemotherapy for metastatic LCNEC ranges from small cell lung carcinoma (SCLC) regimens to nonsmall cell lung carcinoma (NSCLC) chemotherapy regimens. We analyzed outcomes of chemotherapy treatments for LCNEC.

The Netherlands Cancer Registry and Netherlands Pathology Registry (PALGA) were searched for patients with stage IV chemotherapy-treated LCNEC (2003–2012). For 207 patients, histology slides were available for pathology panel review. First-line platinum-based combined chemotherapy was clustered as "NSCLC-t", comprising gemcitabine, docetaxel, paclitaxel or vinorelbine; "NSCLC-pt", with pemetrexed
treatment only; and "SCLC-t", consisting of etoposide chemotherapy.

A panel review diagnosis of LCNEC was established in 128 out of 207 patients. NSCLC-t chemotherapy was administered in 46% (n=60), NSCLC-pt in 16% (n=20) and SCLC-t in 38% (n=48) of the patients. The median (95% CI) overall survival for NSCLC-t chemotherapy was 8.5 (7.0–9.9) months, significantly longer than patients treated with NSCLC-pt, with a median survival of 5.9 (5.0–6.9) months (hazard ratio 2.51, 95% CI 1.39–4.52; *P*=0.002) and patients treated with SCLC-t chemotherapy, with a median survival of 6.7 (5.0–8.5) months (hazard ratio 1.66, 95% CI 1.08–2.56; *P*=0.020).

In patients with LCNEC, NSCLC-t chemotherapy results in longer overall survival compared to NSCLC-pt and SCLC-t chemotherapy.

Introduction

Pulmonary large cell neuroendocrine carcinoma (LCNEC) is a subtype of lung cancer with neuroendocrine morphology, neuroendocrine differentiation on immunohistochemistry, a high mitotic rate (>10 mitosis·2 mm^{-2}) and nonsmall cell cytological features[1]. LCNEC is rare and accounts for ~3% of all lung cancers, but the proportion of lung cancers diagnosed as LCNEC appears to be increasing[2]. Because the histological features of LCNEC overlap with nonsmall cell lung carcinoma (NSCLC) and occasionally with small cell lung carcinoma (SCLC), histological diagnosis can be difficult[3,4].

Because of the difficulties in diagnosing LCNEC, and its rarity, the optimal systemic treatment has not been adequately established[5]. In the current European Society for Medical Oncology (ESMO) guidelines for NSCLC, no specific treatment for LCNEC is described[6]. In the American Society of Clinical Oncology (ASCO) guideline, either platinum–etoposide chemotherapy treatment (SCLC type) or the same regimen as for nonsmall cell nonsquamous carcinoma (NSCLC type) is advised for LCNEC. However, SCLC-type chemotherapy is considered by expert opinion to be most appropriate[7].

Several observations suggest that LCNEC should respond best to a SCLC-type treatment. For instance, recent studies show that the genomic profile of LCNEC corresponds closely with SCLC[8,9]. In addition, we reported that the prognosis and metastatic pattern at diagnosis of LCNEC significantly overlaps with SCLC[10,2]. However, important differences in the response to SCLC-type chemotherapy treatment for LCNEC and SCLC have been reported[5]. Two single-arm phase II trials in LCNEC (n=29 and n=30) showed an objective response rate (ORR) for etoposide or irinotecan combined with cisplatin ranging from 31% to 47%[11,12], substantially lower compared to SCLC phase III trials evaluating etoposide–cisplatinum chemotherapy (ORR ≈66%)[13]. Because of the reported higher resistance to SCLC-type chemotherapy in LCNEC, some clinicians favor a NSCLC-type chemotherapy treatment.

Because of these perceived differences, we investigated the chemotherapy treatment of patients with metastatic LCNEC in the Netherlands from 2003 to 2012. Furthermore, we retrospectively correlated the overall survival and progression free survival (PFS) with chemotherapy type in patients with a panel-reviewed histological diagnosis of LCNEC.

Materials and methods

Data sources and ethical regulations

Data were retrieved from the Netherlands Cancer Registry and Netherlands Pathology Registry (PALGA, the nationwide registry of pathology in the Netherlands[14]). The study was performed according to the cancer registry and pathology registry guidelines and national privacy regulations and approved by the medical ethical committee of the Maastricht University Medical Center (METC azM/UM 14-4-043, November 20, 2014).

Patient selection

All patients with a diagnosis of stage IV LCNEC recorded in either the cancer registry or the pathology registry between January 1, 2003 and December 31, 2012 were included. To select LCNEC from the cancer registry the International Classifications of Disease – Oncology 3rd edition code M8013 was used. Previously we have observed that a wide range of diagnostic terms are used to describe LCNEC[15]. To identify additional LCNEC cases in the cancer registry that had been diagnosed with alternative nomenclature, the additional diagnostic codes M8246 (neuroendocrine carcinoma) and M8574 (NSCLC with neuroendocrine differentiation) were included. Digital summaries of pathology reports retrieved from the pathology registry were screened for the diagnosis of LCNEC, as previously reported[15]. Patients diagnosed with metastatic LCNEC, including patients with tumors diagnosed with a nomenclature possibly referring to LCNEC, treated by chemotherapy retrieved from either of the national databases, were included. Data on the type of chemotherapy treatment was retrospectively updated in 2015 by qualified cancer registry data managers. Patients were excluded if details on chemotherapy were unavailable.

First, we analyzed the type of chemotherapy in the selected patient study group (aim 1). We then performed a pathology review for all patients. Patients with a diagnosis based on cytology and patients for whom the original histopathological slides could not be retrieved were excluded. Overall survival and PFS were determined in patients with a panel-confirmed diagnosis of LCNEC (aim 2).

Data collection

Collected data included stage (tumor, node and metastasis (TNM) stage 6 or 7) and time from diagnosis to death or last follow-up censored for 36 months of overall survival. PFS was calculated from date of diagnosis until first evidence of progression, death or last day of follow-up. Treatment data included chemotherapy subtype, number of

chemotherapy cycles and second-line treatment. First-line chemotherapy was clustered into three groups, as follows. 1) “NSCLC chemotherapy type” (NSCLC-t), consisting of gemcitabine, docetaxel, paclitaxel or vinorelbine; 2) “pemetrexed NSCLC type” (NSCLC-pt), with pemetrexed treatment only; and 3) “SCLC type” (SCLC-t), consisting of etoposide chemotherapy. The platinum components were either cisplatin or carboplatin. Metastatic sites at diagnosis were retrieved from documented clinical data (cTNM). Pathology data included pathology history, pathological specimen type and diagnosis according to the digital pathology report summary.

Pathology revision

Tumor histology slides were collected and included at least one immunohistochemical (IHC) neuroendocrine stain (CD56/NCAM, chromogranin-A or synaptophysin) and a hematoxylin and eosin stained slide. Review was performed by three pathologists (E. Thunnissen, R. van Sulyen and M. den Bakker), who were blinded for clinical outcome and original diagnosis. IHC staining patterns for neuroendocrine markers, cytokeratin’s, TTF1 and p63 and Ki-67 (if available) were assessed by J. Derks and R. van Sulyen prior to the central review meetings. The assessors evaluated hematoxylin and eosin slides at the multihead microscope; information on IHC expression patterns was provided (J. Derks). LCNEC was established when at least two pathologists agreed on the diagnosis, referred to as panel-consensus LCNEC. World Health Organization (WHO) 2015 criteria were evaluated for all panel-consensus established diagnoses. Additional detailed pathology review information can be found in the online supplementary pathology data file.

Statistical analysis

The Chi-squared and Fisher exact tests were used to compare categorical data. Continuous variables were tested using the Mann–Whitney U-test and the median and interquartile range (IQR) reported. Overall survival and PFS censoring took place at the closing date (February 1, 2014). Overall survival was estimated according to the Kaplan–Meier method and tested using the log-rank test. Multivariate Cox regression analysis was performed including covariates significant at univariate analysis. Nonproportionality was visually assessed by log minus log plots. Two-sided *P*-values <0.05 were considered significant. Analyses were performed using SPSS (version 22 for Windows; Chicago, IL, USA).

Results

Population based changes in chemotherapy treatment over time

Data from 1627 patients from the cancer registry and 1172 patients from the pathology registry were retrieved. 355 patients had stage IV disease treated with chemotherapy. After excluding patients for who details of chemotherapy treatment could not be retrieved, chemotherapy treatment was analyzed in 294 patients (Figure 9.1). A complete overview of retrieved diagnoses and chemotherapy treatment is presented in Supplementary Table S9.1. NSCLC-t chemotherapy treatment in LCNEC significantly decreased over time from 59% (2003–2009) to 31% (2010–2012) ($p<0.001$); NSCLC-pt chemotherapy type increased from 10% to 16% ($P=0.29$); and the SCLC type increased from 31% to 53% ($P=0.002$).

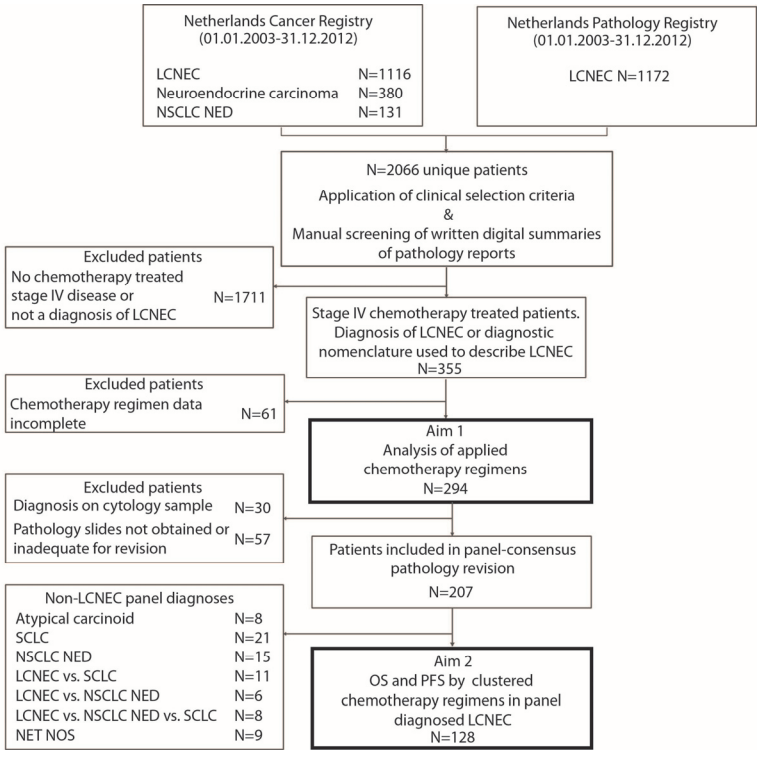

Figure 9.1 CONSORT diagram. Inclusion of patients and the performed pathology review.
Abbreviations: N, number; LCNEC, large cell neuroendocrine carcinoma; NSCLC NED, non-small cell lung carcinoma with immunohistochemically neuroendocrine differentiation; SCLC, small cell lung carcinoma; OS, overall survival; PFS, progression free survival; NET NOS, neuroendocrine tumor not otherwise specified.

Panel-consensus diagnosed LCNEC

Histopathological slides were retrieved from 207 patients. In 128 patients LCNEC was diagnosed by consensus, with 108 cases meeting all required WHO 2015 criteria (Supplementary Table S9.2)[16]. Patients with a panel-confirmed LCNEC diagnosis (n=128) had a median age of 65 (56–71) years, 59% were male and 67% were diagnosed by a (core) needle biopsy specimen (Table 9.1).

Table 9.1 Clinical characteristics of patients with panel-consensus LCNEC (N=128)

Clinical characteristic	Total cohort	Chemotherapy clusters			NSCLC-t versus	
	Total	NSCLC-t	NSCLC-pt	SCLC-t	NSCLC-pt	SCLC-t
	N (%)	*N (%)*	*N (%)*	*N (%)*	*P-value*	*P-value*
Patients	128 (100)	60 (46)	20 (16)	48 (38)	-	-
Age (median, IQR)	65 (56-71)	64 (56-69)	70 (57-74)	63 (55-70)	0.24[i]	0.88[i]
Gender (males)	75 (59)	33 (55)	12 (60)	30 (63)	0.70	0.43
Number of organs with metastases					0.67†*	0.012†
1	61 (48)	29 (48)	14 (70)	18 (38)		*
2	44 (34)	24 (40)	5 (25)	15 (31)		
3	11 (9)	2 (3)	0 (0)	9 (19)		
>3	12 (9)	5 (8)	1 (5)	6 (13)		
Organ metastases at diagnosis						
Bone	34 (27)	14 (23)	5 (25)	15 (31)	0.88	0.34
Liver	68 (53)	30 (50)	10 (50)	28 (58)	1.00	0.39
Brain	17 (13)	7 (12)	2 (10)	8 (17)	0.57	0.81
Adrenal gland	21 (16)	9 (15)	2 (10)	10 (21)	0.57	0.43
Lung	16 (13)	10 (17)	2 (10)	4 (8)	0.47	0.20
Pleura	2 (2)	1 (2)	0 (0)	1 (2)	1.00*	1.00*
Lymph node	28 (22)	14 (23)	4 (20)	10 (21)	0.76	0.76
Non-clustered subtype of CT					-	-
Gemcitabine	46 (36)	46 (76)	-	-		
Paclitaxel	7 (5)	7 (12)	-	-		
Docetaxel	6 (5)	6 (10)	-	-		
Vinorelbine	1 (1)	1 (2)	-	-		
Etoposide	48 (38)	-	-	48 (100)		
Pemetrexed	20 (16)	-	20 (100)	-		
Cycles of chemotherapy					0.30‡	0.09‡
1	18 (14)	6 (10)	2 (10)	10 (21)		
2	15 (12)	5 (8)	4 (20)	6 (13)		
3	14 (11)	6 (10)	3 (15)	5 (10)		
4	63 (49)	30 (50)	11 (55)	22 (46)		
>4	16 (13)	11 (18)	0 (0)	5 (10)		
Data lacking	2 (2)	2 (3)	0 (0)	0 (0)		
Additional chemotherapy						
Second line	29 (23)	13 (22)	4 (20)	12 (25)	0.88	0.68
Third line	6 (5)	3 (5)	1 (5)	2 (4)	1.00*	1.00*

[i] Tested with Mann Whitney U test; † Compared ≤2 organ metastases with >2 organ metastases; ‡ Compared ≤2 cycles versus ≥3 cycles of chemotherapy, excluding unknown cases; * Tested with Fisher Exact test

Abbreviations: IQR, interquartile range; N, Number; CT, chemotherapy; NSCLC-t, cluster of gemcitabine, paclitaxel, docetaxel and vinorelbine chemotherapy; NSCLC-pt, cluster of pemetrexed chemotherapy; SCLC-t; cluster of etoposide chemotherapy

Metastases in the liver (53%), bone (27%) and nonmediastinal lymph nodes (22%) were most common. Metastases confined to a single organ were present in 48% of patients. A minimum of four chemotherapy cycles (median (IQR) 4 (2–4)) were administered in 62% of patients. Second-line chemotherapy was administered in 23% of patients. Patients with more than three metastases in different organs more frequently received SCLC-t chemotherapy. Overall, NSCLC-t chemotherapy was administered in 46% of patients, mainly platinum–gemcitabine (76% of NSCLC-t patients). NSCLC-pt and SCLC-t chemotherapy was administered in 16% and in 38% of patients, respectively. Characteristics of panel-consensus diagnosed LCNEC patients who fulfilled all required WHO criteria were not different and are described in Supplementary Table S9.3.

Overall survival in panel-consensus diagnosed LCNEC by chemotherapy cluster

All but three patients died during the follow-up period. The median (95% CI) overall survival was 7.3 months (6.3–8.2 months). Patients treated with NSCLC-t chemotherapy had a median overall survival of 8.5 months (7.0–9.9 months), which was significantly longer than for patients treated with NSCLC-pt chemotherapy (5.9 months, 5.0–6.9 months; *P*=0.011), and significantly longer than patients treated with SCLC-t chemotherapy (6.7 months, 5.0–8.5 months; *P*=0.012) (Figure 9.2a). In multivariate analysis, including the covariates significant at univariate analyses (sex, age, liver metastasis and number of organs with metastases at diagnosis) (Supplementary Figure S9.2), results remained significant for NSCLC-t versus NSCLC-pt treatment (hazard ratio (HR) 2.51, 95% CI 1.39–4.52; *P*=0.002), and for NSCLC-t versus SCLC-t treatment (1.66, 1.08–2.56; *P*=0.020) (Figure 9.3). Cisplatinum versus carboplatinum compounds did not have a significant effect on the treatment outcome data (Supplementary Figure S9.3). Corresponding results for overall survival and PFS in 108 patients with LCNEC in whose tumor samples all WHO 2015 criteria were confirmed are described in Supplementary Figures S9.3, S9.4 and S9.5.

Overall survival in panel-consensus LCNEC according to chemotherapy subtype

Patients treated with platinum–gemcitabine chemotherapy had a median overall survival (95% CI) of 7.8 months (5.9–9.6 months), which was significantly longer than for platinum–pemetrexed (5.9 months, 5.0–6.9 months; *P*=0.019) and for platinum–etoposide chemotherapy (6.7 months, 5.0–8.5 months; *P*=0.035) (Figure 9.2b). In multivariate analyses overall survival for gemcitabine was superior to pemetrexed chemotherapy (HR 2.39, 95% CI 1.31–4.35; *P*=0.004) and a strong trend was observed

compared to etoposide (1.54, 0.97–2.43; *P*=0.066) (Figure 9.3). Paclitaxel-treated patients had a median overall survival of 8.7 months (95% CI 2.7–14.7 months), significantly longer than for pemetrexed chemotherapy (*P*=0.034), and a strong trend was observed for etoposide chemotherapy (*P*=0.057) (Figure 9.2b). In multivariate analysis paclitaxel showed superior overall survival compared to pemetrexed chemotherapy (HR 4.04, 95% CI 1.46–11.22; *P*=0.007) and etoposide chemotherapy treatment (HR 2.60, 95% CI 1.07–6.35; *P*=0.035) (Figure 9.3).

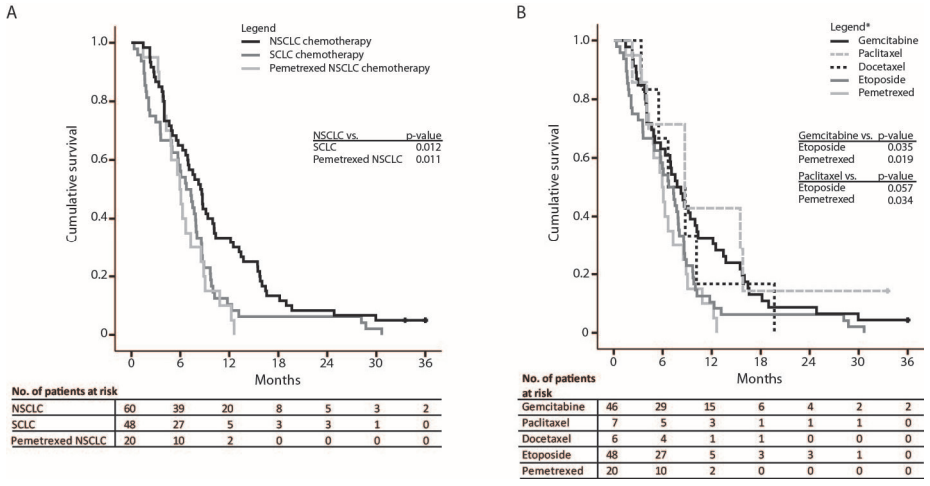

Figure 9.2 **A**) Overall survival in panel-consensus LCNEC (N=128) compared for the chemotherapy clusters and **B**) for subtypes of chemotherapy.
** Excluded Vinorelbine.*
Abbreviations: No, number of; LCNEC, Large cell neuroendocrine carcinoma; SCLC-t, small cell lung carcinoma chemotherapy regimen of platinum-etoposide; NSCLC-t, non-small cell lung carcinoma chemotherapy regimen cluster of platinum and gemcitabine, paclitaxel, docetaxel or vinorelbine; NSCLC-pt, NSCLC regimen of platinum-pemetrexed.

PFS in panel-consensus LCNEC according to chemotherapy subtype

Data on PFS were available in 119 patients; all except one patient progressed or died during the study period. The median PFS (95% CI) was 4.7 months (4.2–5.3 months). Only NSCLC-pt chemotherapy treated patients had a significantly worse PFS (4.1 months, 3.8–4.5 months; *P*=0.040) compared to patients treated with NSCLC-pt chemotherapy (Figure 9.4a). Patients treated with gemcitabine chemotherapy had a significantly longer PFS of 5.2 months (4.1–6.2 months) compared to patients treated with NSCLC-pt chemotherapy (*P*=0.034) (Figure 9.4b). All other comparisons of specific subtypes of chemotherapy showed no significant differences in PFS.

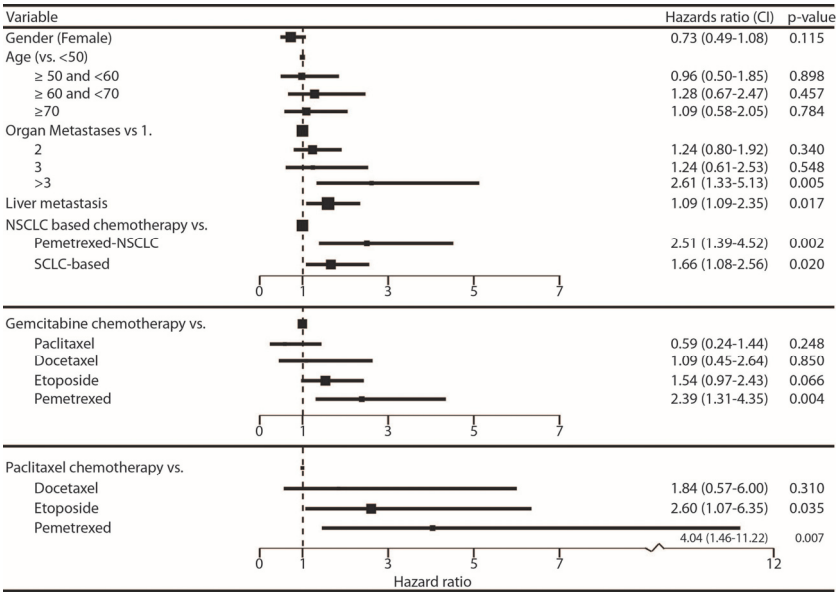

Figure 9.3 Three multivariate models are presented for clustered chemotherapy, platinum-gemcitabine, and platinum-paclitaxel chemotherapy in panel-consensus LCNEC (N=128)
** Excluded Vinorelbine*
Abbreviations: HR, hazard ratio; LCNEC, Large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma

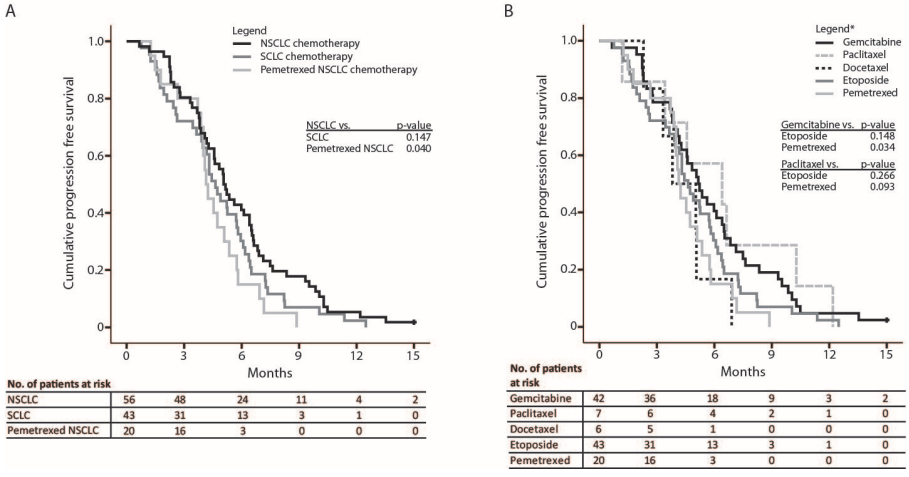

Figure 9.4 **A)** Progression free survival compared for the chemotherapy clusters and **B)** for subtypes of chemotherapy in panel-consensus LCNEC (N=119)
** Excluded Vinorelbine*
Abbreviations: No, number of; LCNEC, Large cell neuroendocrine carcinoma; SCLC-t, small cell lung carcinoma chemotherapy regimen of platinum-etoposide; NSCLC-t, non-small cell lung carcinoma chemotherapy regimen cluster of platinum and gemcitabine, paclitaxel, docetaxel or vinorelbine; NSCLC-pt, NSCLC regimen of platinum-pemetrexed

Discussion

Patients treated with doublet combined chemotherapy for metastatic LCNEC have a poor survival and the optimal chemotherapy treatment for LCNEC remains unascertained. Here we report that patients treated with NSCLC-t chemotherapy, mainly gemcitabine, have superior overall survival compared with patients treated with NSCLC-pt chemotherapy. In addition, the combination of NSCLC-t regimens, excluding those containing pemetrexed, showed superior survival compared with etoposide (SCLC-t) chemotherapy. These results contrast with the advised treatment in the ASCO guideline[7].

Chemotherapy treatment for patients with LCNEC changed significantly between 2003 and 2012 in the Netherlands, with a decrease in NSCLC-t chemotherapy and an increase in SCLC-t chemotherapy. This observation corresponds with data accrued from a 2014 questionnaire survey circulated among 21 Dutch pulmonary physicians. In this survey the majority of physicians (80%) would treat LCNEC with SCLC chemotherapy (Supplementary Figure S9.6). We were unable to find specific explanations why the treatment of LCNEC has changed. Treatment preferences may have been influenced by a study published in 2005 describing the favorable response of LCNEC to SCLC-type chemotherapy[17].

Several studies have evaluated chemotherapy in LCNEC, but the reported studies are heterogeneous in case selection and confirmation of the pathology diagnosis (Table 9.2). Two phase II trials, both with pathology review, have been reported. A European trial[11] reported a median overall survival of 8.0 months (95% CI 3.7–7.9 months), a PFS of 5.0 months (95% CI 4.0–7.9 months) and an ORR of 34% in 29 patients treated with platinum–etoposide chemotherapy. In a Japanese trial [12], a median overall survival of 12.6 (95% CI 9.3–16.0) months, PFS of 5.8 (95% CI 3.8–7.8) months and an ORR of 47% was reported for treatment with platinum–irinotecan (n=30). In retrospectively evaluated cohorts of LCNEC patients, the reported ORR for platinum–etoposide chemotherapy ranged from 37% to 73% and overall survival ranged from 8.4 to 16.5 months[17-20]. Treatment outcomes for SCLC- and NSCLC-type chemotherapy for LCNEC has previously been evaluated; 27 patients showed an improved survival for platinum–etoposide chemotherapy compared to a combination of NSCLC regimens[17]. Conversely, evaluation of an additional 26 patients showed a significantly lower overall survival for platinum–etoposide chemotherapy compared to a combination of NSCLC regimens[19]. Because NSCLC regimens are frequently combined for analysis, there is a lack of data on subtype-specific overall survival and PFS. The reported

ORRs for platinum combined with gemcitabine, docetaxel and paclitaxel are 41% (n=17), 77% (n=9) and 81% (n=11), respectively[18,21].

Table 9.2 Overview of response to chemotherapy in advanced stage LCNEC disease. Studies including patients treated with chemo radiotherapy are not shown

Author	Design	Panel review (number of pathologists)	Inclusion period	NSCLC chemotherapy			SCLC chemotherapy		
				Number	*ORR*	*OS*	*Number*	*ORR*	*OS*
Treut	P	Yes (?)	2004-2009	-	-	-	29	34%	8.0
Niho	P	Yes (6)	2005-2011	-	-	-	30	47%	12.6
Metro	R	No revision	U.A.	-	-	-	37	43%	8.4
Naidoo	R	Yes (3)	2006-2013	11*	0%*	19.5	26**	37%	8.3
Sun	R	Yes (?)	2001-2010	34[i]	50%	9.2	11	73%	16.5
Rossi	R	Yes (3)	1990-2004	15	0%	21	12	50%	51
Fujiwara	R	No (1)	1999-2006	9†	77%	-	13	46%	-
Derks	R	Yes (3)	2003-2012	60 (NSCLC) 20 (pemetrexed-NSCLC)	- -	8.5 5.9	48	33%	6.7

* 4 patients were evaluated according to RECIST, including 2x temezolomide, 1x pemetrexed and 1x platinum combined with Everolimus. ** 19 patients were evaluated according to RECIST criteria. [i] Including gemcitabine-platinum (17), taxane-platinum (4), tyrosine kinase inhibitor (2) and other platinum (11). † Taxane combined with platinum in 7 and taxane monotherapy in 1 patient, 1 patient with platinum-vinorelbine
Abbreviations: (P), prospective; (R), retrospective; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; OS, overall survival in months; U.A. unavailable

Platinum–pemetrexed chemotherapy is advised as first-line treatment in patients with metastatic nonsquamous NSCLC[7]. However, platinum–pemetrexed chemotherapy showed inferior results compared to platinum–etoposide in SCLC[22], a tumor biologically closely related to LCNEC. The poor therapeutic response of pemetrexed may be due to the reported high expression of the thymidylate synthesis (TS) gene
in LCNEC. Increased TS expression is suggested to be related to resistance to pemetrexed therapy[23-25]. The increased tendency for pemetrexed resistance coupled with the reported clinical observations suggests that pemetrexed should not be used in patients with LCNEC.

Molecular changes in LCNEC and SCLC have been described. SCLC is characterized by RB1 and TP53 gene mutations, whereas LCNEC was characterized by mutually exclusive RB1 and TP53 gene inactivation versus a combination of STK11/KRAS/KEAP1 gene mutations[9,26]. In future studies it would be of interest to analyze these patterns to investigate whether the molecular background corresponds with responses to different chemotherapy regimens[9].

This study has several limitations. First, it is a retrospective study and chemotherapy data could not be retrieved in all patients. However, the exclusion of patients was random and not by selection, as evidenced by the similar overall survival and age range of excluded patients compared to the analyzed patient cohort (Supplementary Tables S9.4 and S9.5). Second, information on WHO performance score was lacking, and this may have confounded reported overall survival. We observed no differences in overall survival for treatment with cisplatinum or carboplatinum chemotherapy (Supplementary Figure S9.2). Third, completion of chemotherapy cycles differed slightly between the NSCLC-t and SCLC-t treatments. Nevertheless, up to 62% of patients completed four or more cycles of chemotherapy and this was not significantly different between treatment groups. Fourth, the reported overall survival for chemotherapy-treated subtypes may have been confounded by strong therapeutic effects of second-line treatment. However, in the presented cohort the frequency of second-line treatment was relatively low (23%) and not statistically different among clustered chemotherapy subtypes (Table 9.1). The frequency of second-line treatment is lower than reported in a Japanese phase II trial (86%)[12], but not much lower than reported for daily clinical practice in lung cancer (32%)[27]. Finally, data on PFS were obtained retrospectively and could not be formally evaluated by the RECIST (response evaluation criteria in solid tumors) criteria, as this was analyzed in a real-world setting and not in a clinical trial. Response evaluation was not standardized and incomplete in 40% of patients; therefore, these data are not reported.

In conclusion, we present the largest series of patients with pathology-reviewed metastatic LCNEC to date, and show that NSCLC-t regimens, mainly platinum–gemcitabine chemotherapy, are superior to platinum–pemetrexed and platinum–etoposide treatment. These results need prospective evaluation, ideally in a randomized trial, in centrally confirmed LCNEC.

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Supplemental material

Overview of originally established diagnoses retrieved from digital summaries and patient chemotherapy treatment

An overview of all patients with metastatic disease treated with first line chemotherapy retrieved from the pathology and cancer registry is presented in Table S9.1**.** Confirmed diagnoses from pathology reports included N=164 large cell neuroendocrine carcinoma (LCNEC), N= 48 neuroendocrine carcinomas (NEC), N=65 non-small cell lung carcinoma with neuroendocrine immunohistochemically differentiation (NSCLC NED) and 'other' compromising cases not differentiating between LCNEC and NSCLC NED (N=7). Patients with NEC were more frequently treated with SCLC-t chemotherapy (54%) compared to LCNEC (43%) and NSCLC NED (13%). NSCLC NED was generally treated with NSCLC-t chemotherapy (71%).

Table S9.1 Overview of possible LCNEC diagnoses and treatment overview of chemotherapy

Chemotherapy	LCNEC						NEC						NSCLC NED						Other					
Subtype	*≤2009*		*≥2010*		*Total*		*≤2009*		*≥2010*		*Total*		*≤2009*		*≥2010*		*Total*		*≤2009*		*≥2010*		*Total*	
	N	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*	*N*	*%*
NSCLC-t	45	59	27	31	72	44	13	48	3	14	16	33	35	83	18	55	53	71	2	50	2	67	4	57
SCLC-t	24	31	46	53	70	43	9	33	17	81	26	54	4	10	6	18	10	13	1	25	0	0	1	14
NSCLC-pt	8	10	14	16	22	13	5	19	1	5	6	13	3	7	9	27	12	16	1	25	1	33	2	29

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; NEC, neuroendocrine carcinoma; NSCLC NED, non-small cell lung carcinoma with neuroendocrine immunohistochemically staining; SCLC, small cell lung carcinoma; NSCLC-t, clustered platinum gemcitabine, paclitaxel, docetaxel or vinorelbine chemotherapy; NSCLC-pt, NSCLC platinum-pemetrexed chemotherapy; SCLC-t, SCLC platinum-etoposide chemotherapy

Additional methods for panel-consensus pathology review

Collectively the reviewers systematically scored all cases (RvS, ET, MdB). WHO 2015 criteria were evaluated for each case including presence of neuroendocrine morphology , estimated mitotic activity in non-crushed fields (≤10, 11-30 or >30 /10 high power field (HPF)), and presence of necrosis (none, 'dot-like' [=as occasionally seen in atypical carcinoids] or abundant [= more extensive than 'dot-like']). CD56 was scored as positive when membrane staining was observed. For chromogranin-A and synaptophysin focal small cytoplasmic dots in an occasional tumor cell observed at 40x microscope objective were sufficient for staining (+). Diffuse complete staining observed at overview (4x or 2.5x objective) was scored as strong positive (+++), and in between staining as (++) for all neuroendocrine markers. Finally, when available, p63/p40/TTF1 and cytokeratin staining were evaluated for positivity.
In small tissue samples (<2 mm^2), evaluation of mitoses was performed on the maximal number of assessable high power fields. Counting mitoses was not possible in some cases (Thunnissen et al. JTO 2016, DOI 10.1016/j.jtho.2016.12.004). When available, the MIB1 (Ki-67) staining was scored (<25%, >25%). Either >10 mitosis/10 HPF, abundant tumor necrosis or a Ki-67 staining of more than 25% of tumor cells was sufficient to score for high-grade tumor disease (Rindi et al. 2014 ERC, DOI 10.1530/ERC-13-0246).

All diagnoses established by panel consensus as LCNEC required staining for at least one neuroendocrine marker and were analyzed for OS and PFS (Figure 9.2-9.4, Table S9.2). Occasionally LCNEC was diagnosed despite the absence of neuroendocrine morphology (N=19) or evidence of high-grade disease (N=1) as this could not be observed; mainly because of a limited availability of vital tumor tissue (Table S9.2). Therefore, we additionally analyzed OS and PFS in panel-consensus classified LCNEC for which all required World Health Organization (WHO) 2015 criteria were evaluable (Figure S9.4-9.6, Table S9.2).

Table S9.2 Overview of panel consensus diagnosis, scoring of WHO criteria and original diagnosis

Panel-Consensus diagnosis	WHO 2015 criteria	Original diagnosis
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	DD LCNEC vs NSCLC NED
LCNEC	Yes	NEC
LCNEC	Yes	NEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC

Table S9.2 (continued)

Panel-Consensus diagnosis	WHO 2015 criteria	Original diagnosis
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	NSCLC NED
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	NEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	NEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	NEC

Table S9.2 (continued)

Panel-Consensus diagnosis	WHO 2015 criteria	Original diagnosis
LCNEC	Yes	LCNEC
LCNEC	Yes	NSCLC NED
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	Yes	LCNEC
LCNEC	No	LCNEC
LCNEC	No	LCNEC
LCNEC	No	NSCLC NED
LCNEC	No	LCNEC
LCNEC	No	LCNEC
LCNEC	No	DD LCNEC vs NSCLC NED
LCNEC	No	LCNEC
LCNEC	No	LCNEC
LCNEC	No	NSCLC NED
LCNEC	No	LCNEC
LCNEC	No	NSCLC NED
LCNEC	No	LCNEC
LCNEC	No	LCNEC
LCNEC	No	NEC
LCNEC	No	NSCLC NED
LCNEC	No	LCNEC
LCNEC	No	LCNEC
LCNEC	No	LCNEC
LCNEC	No	LCNEC
LCNEC	No	LCNEC

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; NEC, neuroendocrine carcinoma; NSCLC NED, non-small cell lung carcinoma with neuroendocrine immunohistochemically staining; DD differential diagnosis

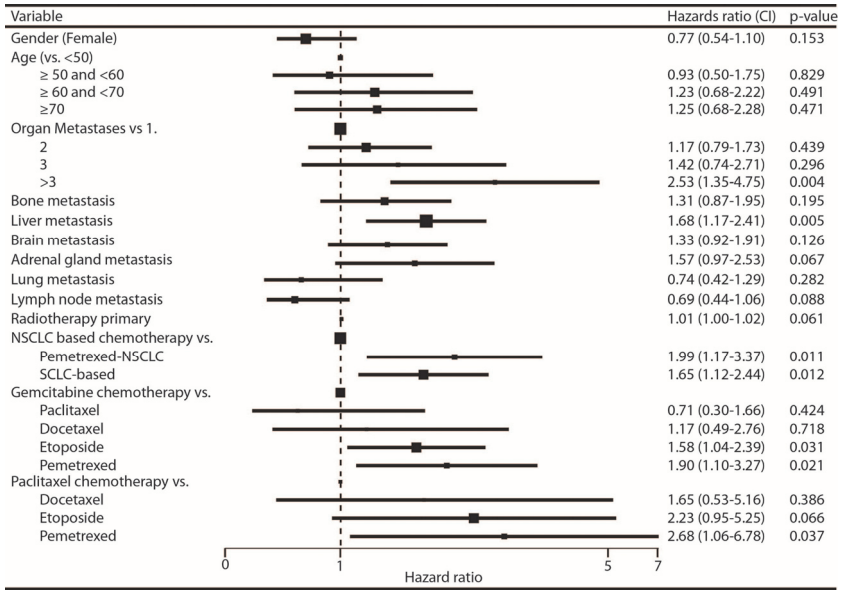

Figure S9.1 Univariate analysis of covariates for overall survival in panel-consensus LCNEC (N=128)
Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma

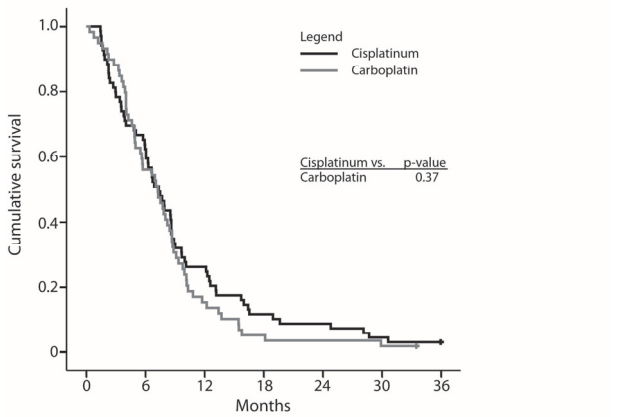

Figure S9.2 Overall survival for panel-consensus LCNEC (N=128) according to treatment by cisplatinum or carboplatinum doublet chemotherapy

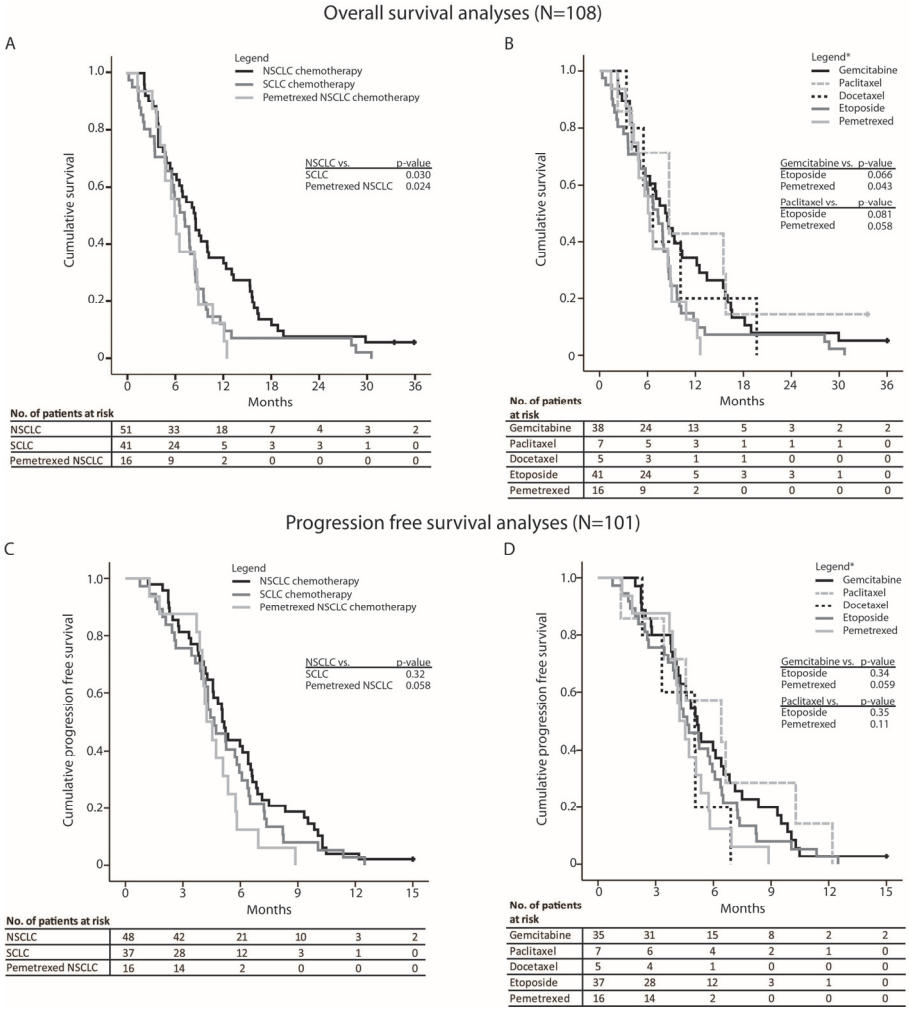

Figure S9.3 **A**) Overall survival in LCNEC for which all WHO 2015 criteria were evaluable (N=108) compared for the chemotherapy clusters and **B**) for subtypes of chemotherapy. **C**) Progression free survival compared for the chemotherapy clusters and **D**) for subtypes of chemotherapy (N=101)
** Excluded Vinorelbine*
Abbreviations: No, number of; LCNEC, Large cell neuroendocrine carcinoma; SCLC-t, small cell lung carcinoma chemotherapy regimen of platinum-etoposide; NSCLC-t, non-small cell lung carcinoma chemotherapy regimen cluster of platinum and gemcitabine, paclitaxel, docetaxel or vinorelbine; NSCLC-pt, NSCLC regimen of platinum-pemetrexed

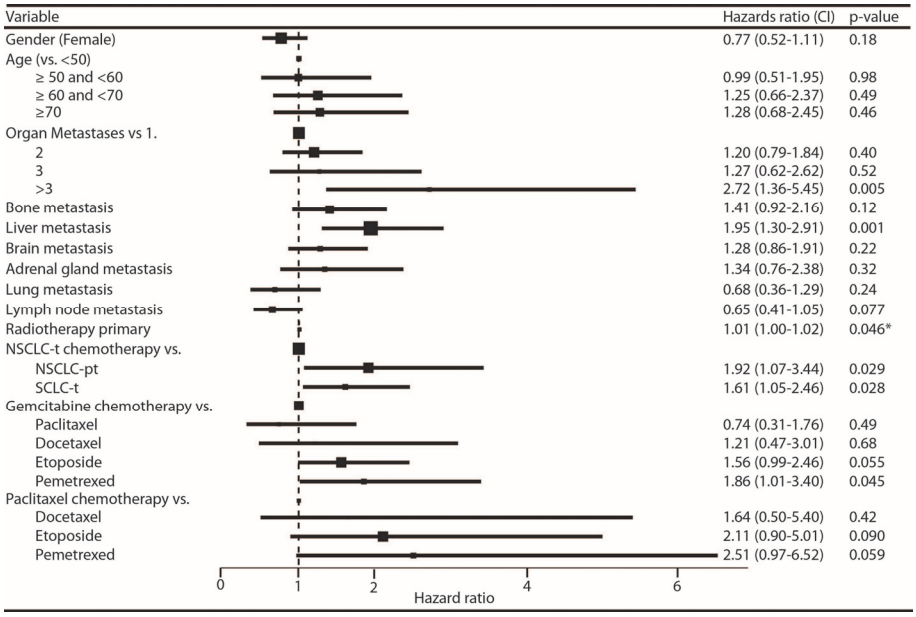

Figure S9.4 Univariate analysis of covariates for overall survival in panel-consensus LCNEC for which all WHO 2015 criteria were evaluable (N=108)

* Excluded for multivariate analyses due to small effect size

Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; WHO, World Health Organization

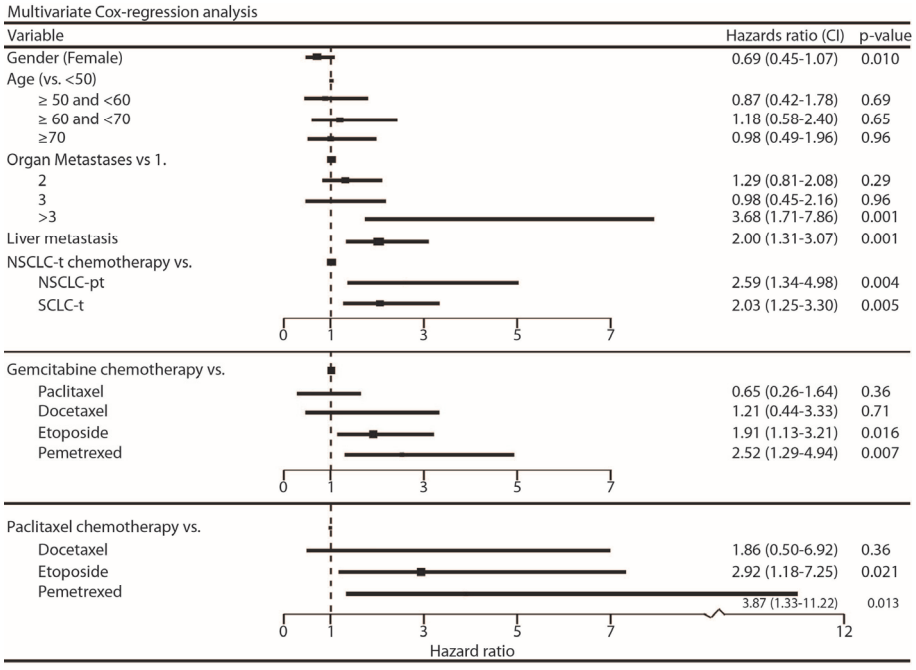

Figure S9.5 Multivariate analysis of overall survival in panel-consensus LCNEC for which all WHO 2015 criteria were evaluable (N=108)
Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; WHO, World Health Organization

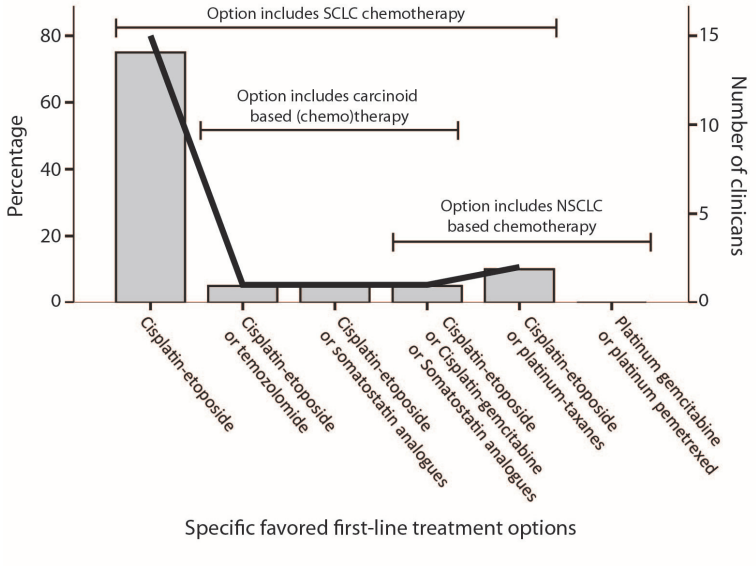

Figure S9.6 Overview of a questionnaire survey among Dutch physicians (*N*=21) on favored first-line treatment in a patient diagnosed with stage IV LCNEC disease based on liver metastases. This questionnaire was circulated during an educational lung cancer meeting "Wengen op de Wadden 2014"
Abbreviations: LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma

Table S9.3 Clinical characteristics of patients with panel-consensus LCNEC meeting all WHO 2015 criteria (N=108)

	Total cohort	Chemotherapy clusters			NSCLC-t versus	
Clinical characteristic	Total	NSCLC-t	NSCLC-pt	SCLC-t	NSCLC-pt	SCLC-t
	N (%)	*N (%)*	*N (%)*	*N (%)*	*P-value*	*P-value*
Patients	108 (100)	51 (47)	16 (15)	41 (38)	-	-
Age (median, IQR)	65 (55-71)	65 (55-69)	68 (57-74)	63 (54-71)	0.62[i]	0.82[i]
Gender (males)	61 (57)	28 (55)	8 (50)	25 (61)		
Number of organs with metastases					1.00†*	0.04†
1	48 (45)	22 (43)	10 (62)	16 (39)		
2	41 (38)	23 (45)	5 (31)	13 (32)		
3	9 (8)	1 (2)	0 (0)	8 (19)		
>3	10 (9)	5 (10)	1 (6)	4 (10)		
Organ metastases at diagnosis						
Bone	31 (29)	12 (24)	5 (31)	14 (34)	0.53	0.26
Liver	62 (57)	28 (55)	10 (63)	24 (59)	0.59	0.72
Brain	15 (14)	8 (16)	0 (0)	7 (17)	0.18	0.86
Adrenal gland	14 (13)	8 (16)	0 (0)	6 (15)	0.18	0.88
Lung	13 (12)	7 (14)	2 (13)	4 (10)	1.00	0.75*
Pleura	2 (2)	1 (2)	0 (0)	1 (2)	1.00*	1.00*
Lymph node	23 (21)	11 (22)	4 (25)	8 (20)	0.74	0.81
Non-clustered subtype of CT					-	-
Gemcitabine	38 (35)	38 (75)	-	-		
Paclitaxel	7 (7)	7 (14)	-	-		
Docetaxel	5 (5)	5 (10)	-	-		
Vinorelbine	1 (1)	1 (2)	-	-		
Etoposide	41 (38)	-	-	41 (100)		
Pemetrexed	16 (15)	-	16 (100)	-		
Cycles of chemotherapy					0.30‡	0.20‡
1	14 (13)	4 (8)	2 (14)	8 (20)		
2	12 (11)	5 (10)	3 (19)	4 (10)		
3	12 (11)	5 (10)	2 (13)	5 (12)		
4	63 (49)	26 (51)	9 (56)	19 (46)		
>4	15 (14)	10 (20)	0 (0)	5 (12)		
Data lacking	1 (1)	1 (2)	0 (0)	0 (0)		
Additional chemotherapy						
Second line	25 (23)	11 (22)	3 (19)	11 (27)	1.00*	0.56
Third line	5 (5)	3 (6)	0 (0)	2 (5)	1.00*	1.00*

[i] Tested with Mann Whitney *U* test. † Compared ≤2 organ metastases with >2 organ metastases. ‡ Compared ≤2 cycles versus ≥3 cycles of chemotherapy, excluding unknown cases. * Tested with Fisher Exact test

Abbreviations: IQR, interquartile range; N, Number; CT, chemotherapy; NSCLC-t, cluster of gemcitabine, paclitaxel, docetaxel and vinorelbine chemotherapy; NSCLC-pt, cluster of pemetrexed chemotherapy; SCLC-t; cluster of etoposide chemotherapy; WHO, World Health Organization

Table S9.4 Clinical characteristics of patients diagnosed with LCNEC in the routine practice (N=164)

	Total cohort	Chemotherapy clusters			NSCLC-t vs.	
Clinical characteristic	Total	NSCLC-t	NSCLC-pt	SCLC-t	NSCLC-pt	SCLC-t
	N (%)	*N (%)*	*N (%)*	*N (%)*	*P-value*	*P-value*
Patients	164 (100)	72 (44)	22 (13)	70 (43)	-	-
Overall survival (95% CI)	7.6 [6.9-8.3]	7.8 [6.5-9.2]	7.9 [5.3-10.4]	6.8 [5.7-7.9]	0.74	0.17
Progression free survival (95% CI)	5.0 [4.6-5.5]	5.3 [4.5-6.2]	4.6 [3.7-5.5]	4.8 [4.2-5.3]	0.96ǂ	0.41ǂ
Age (Median, IQR)	63 (56-70)	64 (56-70)	69 (58-74)	63 (53-69)	0.81[i]	0.31[i]
Gender (males)	86 (52)	35 (49)	10 (46)	41 (59)	0.80	0.23
Number of organs with metastases					1.00†*	0.008†*
1	71 (43)	33 (46)	14 (64)	24 (34)		
2	59 (36)	30 (42)	5 (23)	24 (34)		
3	20 (12)	4 (5)	2 (9)	14 (20)		
>3	14 (9)	5 (7)	1 (4)	8 (12)		
Organ metastases at diagnosis						
Bone	49 (30)	20 (28)	3 (14)	26 (37)	0.18	0.23
Liver	83 (51)	34 (47)	8 (36)	41 (59)	0.37*	0.18
Brain	34 (21)	12 (17)	5 (23)	15 (21)	0.76*	0.48
Adrenal gland	32 (20)	14 (20)	5 (23)	13 (19)	0.77*	0.90
Lung	25 (15)	12 (17)	1 (5)	12 (17)	0.29*	0.94
Pleura	3 (2)	2 (3)	0 (0)	1 (1)	1.00*	1.00*
Lymph node	28 (17)	13 (18)	8 (36)	7 (10)	0.09	0.17
Non-clustered subtype of CT					-	-
Gemcitabine	57 (35)	57 (79)	-	-		
Paclitaxel	9 (6)	9 (13)	-	-		
Docetaxel	5 (3)	5 (7)	-	-		
Vinorelbine	1 (1)	1 (1)	-	-		
Etoposide	70 (42)	-	-	70 (100)		
Pemetrexed	22 (13)	-	22 (100)	-		
Cycles of chemotherapy					0.35‡*	0.16‡
1	20 (12)	6 (8)	2 (9)	12 (17)		
2	15 (9)	5 (7)	4 (18)	6 (9)		
3	19 (12)	11 (16)	2 (9)	6 (9)		
4	83 (51)	36 (50)	14 (64)	33 (47)		
>4	24 (15)	11 (15)	0 (0)	13 (19)		
Data lacking	3 (2)	3 (4)	0 (0)	0 (0)		
Additional chemotherapy						
Second line	44 (27)	18 (25)	5 (23)	21 (30)	0.83	0.51
Third line	6 (4)	3 (4)	1 (5)	2 (3)	1.00	1.00*

ǂ N=154 patients;[i] Tested with Mann Whitney U test; † Compared ≤2 organ metastases with >2 organ metastases; ‡ Compared ≤2 cycles versus ≥3 cycles of chemotherapy, excluding unknown cases; * Tested with Fisher Exact test

Abbreviations: IQR, interquartile range; N, Number; CT, chemotherapy; CI, 95% confidence interval; ; NSCLC-t, clustered platinum gemcitabine, paclitaxel, docetaxel or vinorelbine chemotherapy; NSCLC-pt, NSCLC platinum-pemetrexed chemotherapy; SCLC-t, SCLC platinum-etoposide chemotherapy.

Table S9.5 Comparison of characteristics of patients with complete and with lacking chemotherapy data diagnosed with LCNEC in the routine pathology practice (N=225)

	Total cohort	Chemotherapy information		
Clinical characteristic	Total	Complete	unavailable	Versus
	N (%)	*N (%)*	*N (%)*	*P-value*
Patients	225 (100)	164 (73)	61 (27)	-
OS (95% CI)	7.9 [7.1-8.7]	7.6 [6.9-8.3]	9.2 [7.7-10.6]	0.16
Age (Median, IQR)	63 (56-70)	63 (56-70)	62 (56-69)	0.50[i]
Gender (males)	130 (58)	86 (52)	44 (72)	0.01
Number of organs with metastases				0.68
1	102 (47)	75 (48)	27 (47)	
2	75 (35)	52 (33)	23 (40)	
3	25 (12)	19 (12)	6 (10)	
>3	13 (6)	11 (7)	2 (3)	

[i] Tested with Mann Whitney U test

Abbreviations: 95% CI, 95% confidence interval; IQR, interquartile range; N, Number; LCNEC, large cell neuroendocrine carcinoma.

Chapter 10

Molecular subtypes of pulmonary large cell neuroendocrine carcinoma predict chemotherapy treatment outcome

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* Authors contributed equally

Abstract

Treatment of large cell neuroendocrine carcinoma (LCNEC) with non-small cell lung carcinoma (NSCLC)-type chemotherapy or small cell lung carcinoma (SCLC)-type chemotherapy is subject of debate. Genomic studies have identified two mutually exclusive molecular subtypes of LCNEC: the *RB1* mutated (mostly co-mutated with *TP53*) and the *RB1* wild-type group. We assessed if these subtypes have a predictive value on chemotherapy outcome.

Clinical data and tumor specimens were retrospectively obtained from the Netherlands Cancer Registry and Pathology Registry. Panel-consensus pathology revision of original tumor slides was performed on 232 cases. Next-generation sequencing (NGS) for *TP53, RB1, STK11,* and *KEAP1* genes, as well as immunohistochemistry (IHC) for RB1 and P16 was performed and correlated with overall survival (OS) and progression free survival (PFS), stratifying for chemotherapy clustered into NSCLC-GEM/TAX including platinum + gemcitabine or taxanes and SCLC-PE including platinum-etoposide.

LCNEC was consensually diagnosed in 148 cases. Subsequently, 79 and 109 passed the quality controls for NGS and IHC, respectively. *RB1* mutations and loss of expression were detected in 47% (n=37) and 72% (n=78) of the cases. *RB1* wild-type LCNEC treated with NSCLC-GEM/TAX had a significantly longer OS (9.6 [95% CI 7.7-11.6] months) than those treated with SCLC-PE (5.8 [5.5-6.1]; *P*=0.026). Similar results were obtained for patients expressing RB1 in their tumors (*P*=0.001), also for PFS (*P*=0.018). RB1 staining with/or P16 loss showed similar results. No differences were observed in patients with *RB1* mutated or lost RB1 expression.

Patients with LCNEC carrying a wild-type and expressed *RB1* do better with NSCLC-GEM/TAX treatment than with SCLC-PE chemotherapy. However, *RB1* mutated *LCNEC* treated with NSCLC-GEM/TAX do as bad as SCLC-PE. Prospective studies should be initiated.

Introduction

Large-cell neuroendocrine carcinoma (LCNEC) is a high-grade neuroendocrine carcinoma with non-small cell cytological features and has an incidence of 1-3% of lung cancer[1,2]. Similar to small cell lung cancer (SCLC), LCNEC is a disease with a poor prognosis[2,3]. The diagnosis of LCNEC requires assessing of both morphology and neuroendocrine differentiation by immunohistochemistry (IHC)[4,5]. Previously, we and others have shown that separation of LCNEC from SCLC and pulmonary carcinoids can be difficult even on resection specimens[6-10]. In the current WHO classification LCNEC is actually an umbrella term comprising cases overlapping with SCLC, NSCLC and carcinoids with >10 mitosis/ 2 mm[28]. The use of immunohistochemistry may support in the distinction of SCLC and carcinoids.

To improve the separation of LCNEC from carcinoids on a biopsy specimen, the application of the proliferation marker Ki-67 was proposed, with a cut-off >20%[10]; however, diagnostic overlap of LCNEC with SCLC remains an issue for pathologists, which is further worsened by the fact that, at diagnosis, LCNEC is often metastasized and commonly only one biopsy specimen is available[11]. Distinguishing LCNEC from SCLC can be difficult because of I) crush artefacts, II) distorted cytological features of SCLC on larger tissue samples[12], III) tumor heterogeneity[12], and IV) overlap in cell and nuclear size between LCNEC and SCLC[9]. Therefore, markers that aid the diagnostic separation of LCNEC from SCLC or that impact the clinical decision-making are urgently needed.

Chemotherapy treatment for LCNEC is subject of debate since it seems to be less chemo-sensitive than SCLC. In the American Society of Clinical Oncology (ASCO) guideline, either platinum–etoposide chemotherapy treatment or the same regimen as for nonsmall cell nonsquamous carcinoma is advised for LCNEC[13], with platinum-etoposide chemotherapy being considered as the most appropriate[13]. Nevertheless, recent studies indicate that patients with LCNEC have a more favorable outcome when treated with platinum-gemcitabine or taxane chemotherapy (NSCLC-GEM/TAX) compared to platinum-etoposide chemotherapy (SCLC-PE)[14-16]. Also, treatment outcome has never been linked to a specific molecular alteration.

Several next-generation sequencing (NGS) studies have shown that LCNEC tumors can be further subdivided into two mutually exclusive groups based on their mutational patterns[17,18]: one harboring bi-allelic inactivation of *TP53* and *STK11/KEAP1* alterations, and the other one enriched for bi-allelic inactivation of *TP53* and *RB1*, a hallmark of SCLC. It has been hypothesized that these LCNEC subtypes may require different

chemotherapy treatment[17]. We tested if the described molecular LCNEC subtypes are predictive for chemotherapy treatment outcome.

Materials and methods

Regulations

The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre (METC azM/UM 14-4-043) and is performed according to the Dutch "Federa, Human Tissue and Medical Research: Code of conduct for responsible use (2011)" regulations not requiring patient informed consent.

Patient and tumor selection

In this retrospective population-based study all data were retrieved from the Netherlands Cancer Registry and Netherlands Pathology Registry (PALGA, the nationwide registry of pathology in the Netherlands[19]) as described previously[11].
Data managers from the cancer registry retrospectively updated (2015) clinical data of all first-line chemotherapy-treated stage IV LCNEC patients (n=232, Figure 10.1). Available data included clinical characteristics, TNM stage, overall survival (OS), and progression free survival (PFS) from date of diagnosis until first evidence of progression, death or last day of follow-up, and chemotherapy details. All patients received platinum doublet (cisplatin or carboplatin) chemotherapy treatment, further divided into three groups: "NSCLC-GEM/TAX" including gemcitabine, docetaxel, or paclitaxel; "NSCLC-PEM" including pemetrexed; and "SCLC-PE" including etoposide. NSCLC-PEM chemotherapy was separated from the other NSCLC regimens because of previously reported resistance in (large cell) neuroendocrine carcinomas[14,20-23].

Panel consensus pathology revision

From all histological specimens, the original hematoxylin and eosin (HE) and IHC slides were collected. Subsequently, three pathologists (RvS, ET, MdB) who were blinded for clinical outcome and for paired biopsy specimens, systematically scored all cases at a multi-head microscope for WHO 2015 criteria. Proliferative activity was evaluated by estimation of MIB1 and mitotic counting (mitoses / 2 mm^2)[8]. The MIB1 (Ki-67) staining was scored (<25%, >25%) when available[10,24]. Either >10 mitosis/2 mm^2, abundant tumor necrosis, or a Ki-67 staining of >25% of tumor cells was sufficient to score for high-grade tumor[19,24]. Diagnoses were considered as consensus when at least two pathologists agreed, further referred to as panel-consensus. All panel-consensus LCNEC tumors were

included for NGS and IHC staining analysis when formalin fixed paraffin embedded (FFPE) tissue block(s) were available (n=109) (Figure 10.1).

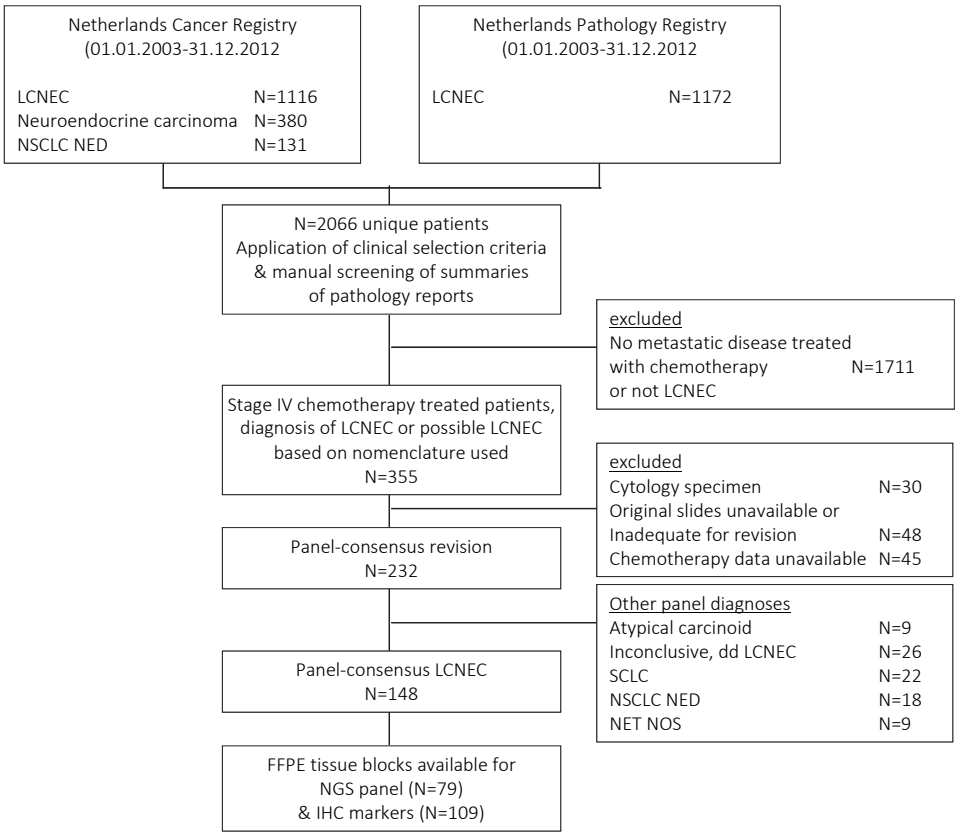

Figure 10.1 Selection of patients and tumor slides for panel-consensus review and molecular analyses
Abbreviations: N, number; LCNEC, large cell neuroendocrine carcinoma; NSCLC NED, non-small cell lung carcinoma with immunohistochemical neuroendocrine differentiation; SCLC, small cell lung carcinoma; NET NOS, neuroendocrine tumor not otherwise specified; NGS, next generation sequencing; IHC, immunohistochemistry

DNA isolation

Tumor macro dissection was performed aiming at a tumor cell content of at least 20%. DNA was extracted from four to eight 10-µm slides using the Maxwell FFPE LEV Automated DNA Extraction Kit (Promega Corporation, Madison, USA). DNA concentration was measured using the QuantiFluor dsDNA Dye System (Promega Corporation, Madison, USA).

Amplicon design and target enrichment

One hundred sixty-nine amplicons of 150 base pairs (bp) in size were designed covering the exons of *TP53* (100%), *RB1* (95%), *STK11* (81%), *KEAP1* (95%) and *MEN1* (90%) with the Qiagen GeneRead DNAseq Custom V2 Builder tool reference CNGHS-02445X-169 (GRCh37). A validated in-house protocol (IARC) was used to perform multiplex PCR further described in the supplementary methods.

Library preparation and next generation sequencing

Library preparation is described in the supplementary methods. Sequencing of the libraries was performed on the Ion Torrent™ Proton Sequencer (Life Technologies Corp., USA) aiming for deep coverage (minimal 250×), using the Ion PI™ Hi-Q™OT2 200 Kit and the Ion PI™ Hi-Q™ sequencing 200 Kit with the Ion PI chip V3 (Life Technologies Corp., USA), following the manufacture's protocols.

Technical duplicates and bioinformatical analysis

Technical duplicates were included for all samples and processed in identical 96 and 384 well plates to prevent PCR errors. Sequencing data was aligned to the hg19 (GRCh37) reference genome and BAM files were generated using the Torrent Suite Software (v4.4.2). Read depth for all amplicon positions were calculated using SAMtools [25] and samples with a median coverage lower than 250x were excluded. Needlestack (revision 1b57abbc92) was used to call variants with default parameters except for the base-quality and the mapping-quality thresholds (10 and 1 respectively)[26]. Annotation was performed with ANNOVAR[27]. Using the PopFreqAll (popfreq_all_20150413), COSMIC v77, SIFT and Polyphen (dbnsfp30a) databases[28,29]. We only considered mutations identified by Needlestack in the two technical duplicates and excluded the ones with an allelic fraction lower than 5%, a relative-variant strand bias (RVSB) higher than 0.85, or already reported as a germline mutation in any of the ExAC, ESP or 1000G populations with a frequency larger than 0.001[30-32]**.** Additionally, all mutations had to be reported in the COSMIC database, or in recently (un)published SCLC/LCNEC studies[18,33], or classified as deleterious by SIFT or Polyphen.

RB1 and P16 immunohistochemistry and scoring

RB1 (13A10, C-terminal) and P16 (JC8) were stained, further described in the supplementary methods. Tonsillar tissue (P16/RB1) and tumor stromal cells (internal control RB1) were included as positive controls. H-scores were calculated as a total score of the percentage of tumor cells with staining intensity 1 (weak nuclear staining) x 1,

intensity 2 (moderate nuclear staining) x2, and intensity 3 (strong nuclear staining) x3 with a maximum score of 300. H-scores were evaluated by EJS who was blinded for all clinical, histopathological, and mutational data.

Statistics

All analyses were performed using SPSS (version 22 for Windows, Inc., Chicago, IL). To compare categorical data χ^2 and Fisher Exact test were used; for continues variables the Wilcoxon rank test was used. OS and PFS were analyzed using two-sided log-rank test and survival curves were estimated using the Kaplan-Meier method. To evaluate the predictive role of RB1 mutation and IHC status, a Cox-regression model was used including an interaction term for the marker and chemotherapy treatment. Two-sided *P*-values <0.05 were considered significant.

Results

Value of the pathology revision and molecular characterization by next-generation sequencing

In total 148 out of the initially 232 LCNEC diagnoses were confirmed as panel-consensus LCNEC tumors (Figure 10.1, pathology data Supplementary Data File Table S10.1). Tumors reclassified as carcinoids (n=9) had longer survival than those confirmed as LCNEC *(P*=0.008), supporting the additive value of the pathology revision (Figure S10.1 A-B). Of the 148 confirmed LCNEC, 79 tumors passed the quality controls for NGS and were, therefore, sequenced (Supplementary Data File Table S10.2).

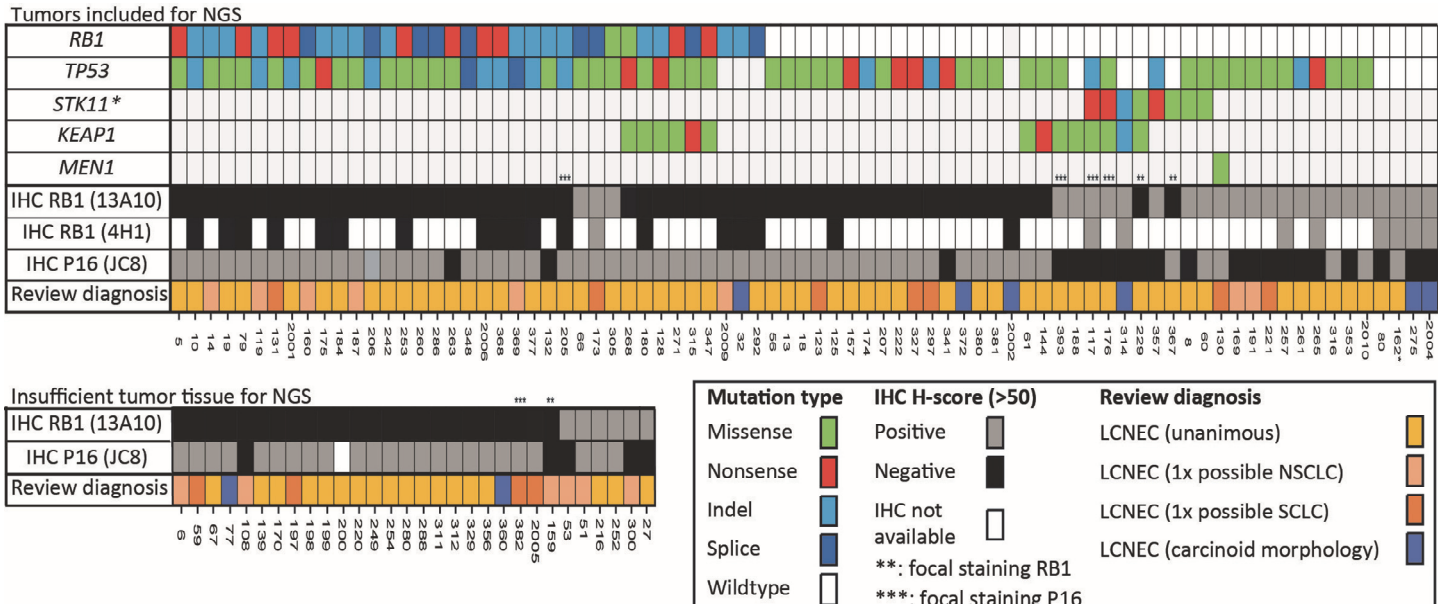

Figure 10.2 Overview of genomic profiles of LCNEC analysed by targeted exon sequencing of the genes *RB1, TP53*, *STK11, KEAP1* and *MEN1* and by immunohistochemistry for RB1 and P16. In total 79 LCNEC tumours were sequence and an additional 30 were analysed by immunohistochemistry only
Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; NGS, next generation sequencing. * In tumour sample 162 the DNA was isolated on tissue obtained 10 months after initiation of treatment

We obtained a median coverage of 2850x (range 261-6870) per sample. A mutation in *TP53* was present in 85% of the cases (n=67), *RB1* in 47% (n=37), *STK11* in 10% (n=8), *KEAP1* in 18% (n=14), and *MEN1* in only one tumor; four samples were wild-type for the 5 genes analyzed (5%) (Figure 10.2). *RB1* was co-altered with *TP53* in 92% of the 37 *RB1* mutated tumors, and mutually exclusive with *STK11* mutations (100%) *P*=0.006 but not with *KEAP1* mutations (57%) *P*=0.71. We did not observe an increased frequency of *RB1* mutations in LCNEC when one out of the three participating reviewing pathologists diagnosed SCLC (n=7, *RB1* mutation in 29% *versus* 49%, *P*=0.44). Hence, the apparent cytological features discriminative of either LCNEC or SCLC for the pathologist in the pathology specimen, does not seem to relate to the *RB1* gene mutation status.

Clinical relevance of the mutational patterns of LCNEC tumors

The clinical characteristics of the patients, which tumors were sequenced, are shown in Table 10.1: median age was 64 (51-79), 65% were males, 55% completed first-line chemotherapy (≥4 cycles), and 19% received second line chemotherapy treatment. In patients with available data on the subtype of chemotherapy (n=72, 91%), we observed that *RB1* wild-type LCNEC patients treated with NSCLC-GEM/TAX showed a significant longer OS compared to SCLC-PE (9.6 [95% confidence interval (CI), 7.7-11.6] *versus* 5.8 [95% CI, 5.5-6.1] months, *P*=0.026) and NSCLC-PEM chemotherapy (6.7 [95% CI, 5.1-8.2], *P*=0.039) (Figure 10.3A). Using a Cox-regression model, only *RB1* wild-type LCNEC showed a significant difference (Hazard ratio (HR) 2.37 (95% CI, 1.09-5.19) favoring NSCLC-GEM/TAX chemotherapy over SCLC-PE treatment. However, comparison of the overall group did not identify a significant interaction between *RB1*-mutation status and chemotherapy treatment (*P*=0.35, Figure 10.4).

Progression-free survival (PFS) of *RB1* wild-type NSCLC-GEM/TAX treated patients were 6.1 [95% CI, 4.2-8.0] months also significantly higher compared to treatment with SCLC-PE (5.7 [95% CI, 3.9-7.6], *P*=0.019) but similar to treatment with NSCLC-PEM chemotherapy (4.7 [95% CI, 3.0-6.4], *P*=0.18) (Figure S10.2A). In *RB1* mutated LCNEC patients, no differences in OS or PFS were observed for different chemotherapy regimens (Figure 10.3D and S10.2D).

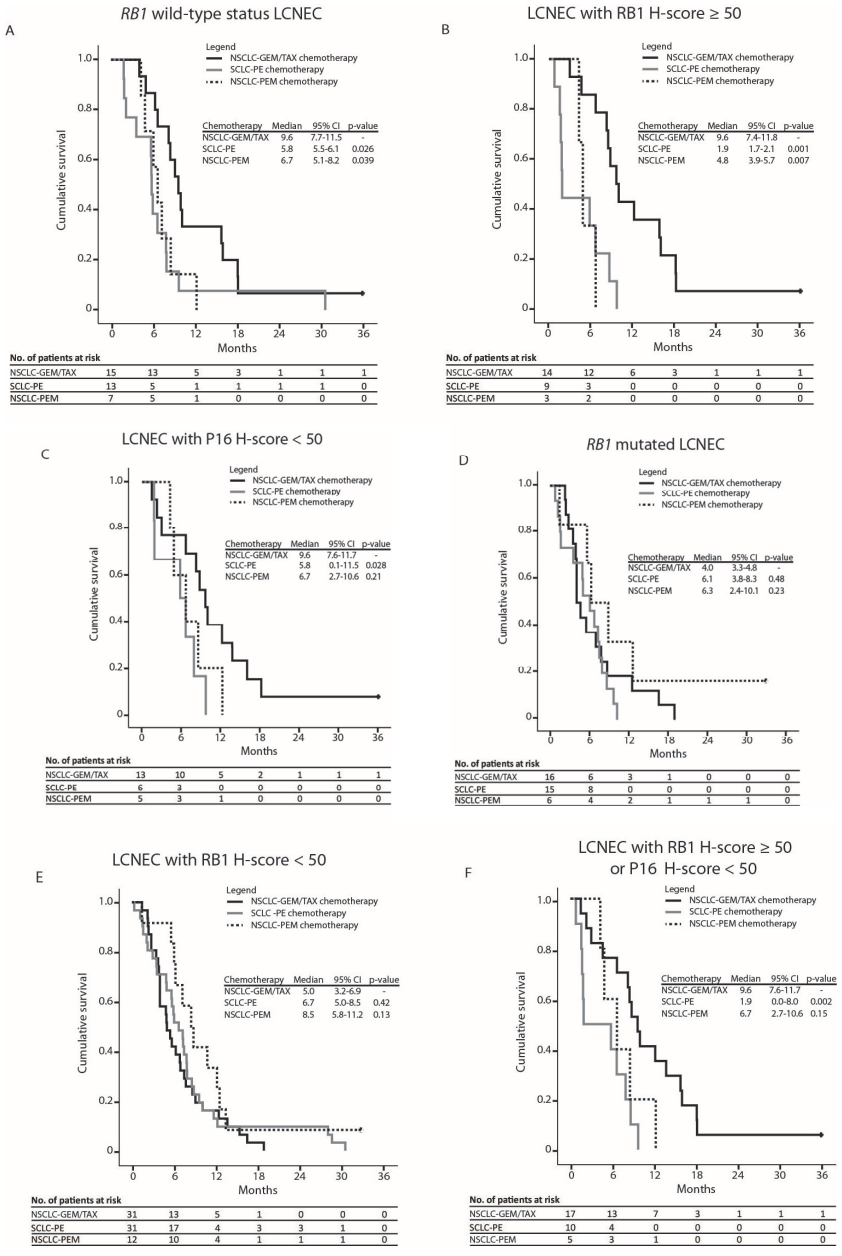

Figure 10.3 Overall survival for subtypes of chemotherapy in consensus LCNEC in **A**) with *RB1* wild-type*; **B**) with an H-score ≥50 for RB1* IHC; **C**) with an H-score <50 for P16 IHC; **D**) with *RB1* mutation; **E**) with an H-score <50 for RB1 IHC; **F**) with an H-score ≥50 for RB1* or <50 for P16 on IHC analysis
Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; NGS, next generation sequencing; No, number of. *Case 162 excluded from analyses

Table 10.1 Clinical characteristics of patients included for NGS and IHC analyses

Clinical characteristics	Total	NGS RB1^{wt}	RB1^{mt}	*P*-value ^{wt} vs. ^{mt}	Total	IHC RB1^{+}	RB1^{-}	*P*-value $^{+}$ vs. $^{-}$
Total patients included (n)	79	42	37		109	31	78	
Age (median, IQR)†	65 (51-79)	64 (46-82)	65 (53-77)	0.87	64 (59-79)	64 (52-76)	63 (48-78)	0.99
Gender				0.37				0.33
Male	51 (65)	29 (69)	22 (59)		66 (61)	21 (68)	45 (58)	
Female	28 (35)	13 (31)	15 (41)		43 (39)	10 (32)	33 (42)	
Chemotherapy clusters				0.91*				0.56*
NSCLC-GEM/TAX	31 (39)	15 (35)	16 (43)		45 (41)	14 (45)	31 (40)	
SCLC-CE	28 (35)	13 (31)	15 (41)		40 (37)	9 (29)	31 (40)	
NSCLC-PEM	13 (17)	7 (17)	6 (16)		15 (14)	3 (10)	12 (15)	
Unknown	7 (9)	7 (17)	0 (0)		9 (8)	5 (16)	4 (5)	
Chemotherapy subtypes				-				-
Gemcitabine	22 (28)	11 (26)	11 (30)		35 (32)	9 (29)	26 (35)	
Taxanes (docetaxel/paclitaxel)	9 (11)	4 (9)	5 (14)		10 (9)	5 (16)	5 (7)	
Etoposide	28 (35)	13 (30)	15 (40)		40 (37)	9 (29)	31 (41)	
Pemetrexed	13 (16)	7 (17)	6 (16)		15 (14)	3 (10)	12 (16)	
Unknown	7 (9)	7 (17)	0 (0)		9 (8)	5 (16)	1 (1)	
Cycles of chemotherapy				0.47**				0.56**
1	9 (11)	5 (12)	4 (11)		17 (16)	5 (16)	12 (15)	
2	13 (17)	5 (12)	8 (22)		13 (12)	2 (7)	11 (14)	
3	12 (15)	5 (12)	7 (19)		14 (13)	5 (16)	9 (12)	
4	34 (43)	20 (47)	14 (37)		49 (45)	13 (42)	36 (46)	
>4	9 (11)	5 (12)	4 (11)		13 (12)	4 (13)	9 (12)	
Unknown	2 (3)	2 (5)	0 (0)		3 (3)	2 (6)	1 (1)	
Second line chemotherapy treatment				0.99				0.21
No	64 (81)	34 (81)	30 (81)		89 (82)	23 (74)	66 (85)	
Yes	15 (19)	8 (19)	7 (19)		20 (18)	8 (26)	12 (15)	

* Excluded unknown cases for comparison. ** Excluded unknown cases for comparison, compared ≤2 vs >2 cycles. † Wilcoxon rank test

Abbreviations: IQR, inter quartile range; NGS, next generation sequencing; IHC, immunohistochemistry; wt, wild-type; mt, mutation; GEM/TAX, platinum-gemcitabine or taxanes chemotherapy; PE, platinum-etoposide chemotherapy; PEM, platinum-pemetrexed chemotherapy

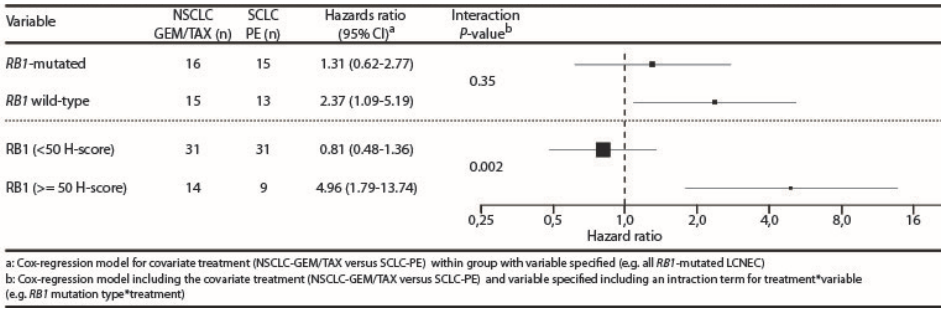

Figure 10.4 Cox-regression model for overall survival including the covariates *RB1* and chemotherapy. A test for interaction was performed to evaluate the predictive value of *RB1* mutations and RB1 immunohistochemical expression for chemotherapy outcome
Abbreviations: NSCLC, non-small cell lung cancer; GEM-TAX, platinum chemotherapy combined with gemcitabine or taxanes; SCLC, small cell lung cancer; PE, platinum-etoposide

Impact of mutational patterns on immunohistochemistry analyses

In *RB1* mutated LCNEC the median H-score for RB1 IHC was 0 (range 0-200) compared to the internal controls. RB1 H-score was 0 in 92% of *RB1* mutated LCNEC; three cases with *RB1* and *TP53* mutations retained expression of RB1 including two splice mutations and one single nucleotide variation all reported in COSMIC (Supplementary Data File Table S10.2). We evaluated if different *RB1* mutations (i.e. splice / indel / single nucleotide variants) showed different results for C and N-terminal protein staining suggestive for alternative protein translation. However, no differences were observed (Figure 10.2). In LCNEC tumors with *RB1* wild-type, the median H-score was 50 (range 0-200). The H-score was ≥50 in 52% of LCNEC with *RB1* wild-type (Figure 10.2, Supplementary Data File Table S10.2 and exemplary case for RB1 staining in Figure S10.3A & C). Still, 48% of *RB1* wild-type LCNEC also had an H-score <50 (median 0, range 0-10). This loss of RB1 IHC suggests alternate mechanisms for *RB1* inactivation.

We also evaluated P16 protein expression because previous studies identified a correlation between *RB1* inactivation and P16 expression and we wanted to investigate the predictive value of RB1 and P16 expression for treatment. In the *RB1* mutated LCNEC the median H-score for P16 protein expression was 300 (range, 0-300); in 95% of RB1 mutated LCNEC, the H-score was ≥200 while two cases were identified with an H-score of 0. The median H-score for *RB1* wild-type tumors was 180 (range, 0-300). In 91% of LCNEC with an H-score <50 for P16, the *RB1* gene was not mutated (Figure 10.2, exemplary P16 staining Figure S10.3B & D).

Based on these results we used RB1 IHC expression with H-score ≥50 as a cut-off to identify LCNEC with an expected functional *RB1* gene. A cut-off <50 was applied for P16 IHC expression.

Patients with LCNEC showing a RB1 H-score ≥50 had a significant longer OS when treated with NSCLC-GEM/TAX compared to SCLC-PE (9.6 [95% CI, 7.4-11.8] months *versus* 1.9 [95% CI, 1.7-2.1] months, *P*=0.001) and NSCLC-PEM (4.8 [95% CI, 3.9-5.7] months, *P*=0.007, Figure 10.3B). Cox-regression analysis confirmed the predictive value of RB1 staining on superior prognosis when treated with NSCLC-GEM/TAX *versus* SCLC-PE chemotherapy (HR 4.96 (95% CI, 1.79-13.74), *P*-value for interaction 0.002, Figure 10.4). Also, PFS was significantly longer in NSCLC-GEM/TAX with 5.5 [95% CI, 1.9-9.0] months *versus* SCLC-PE with 1.7 [95% CI, 0.0-4.8] months (*P*=0.023) but not *versus* NSCLC-PEM with 4.1 [95% CI, 4.0-4.2] months (*P*=0.21, Figure S10.2B). No significant differences were observed in LCNEC with an H-score <50 for RB1 IHC expression (Figure 10.3E and S10.2E).

P16 IHC H-score <50 in LCNEC correlated with improved OS for NSCLC-GEM-TAX *versus* SCLC-PE chemotherapy (*P*=0.028, Figure 10.3C) but with no change in PFS (*P*=0.24, Figure S10.2C). Combined evaluation of RB1 H-score ≥50 and/or P16 <50 showed identical results for OS (*P*=0.002, Figure 10.3F) and PFS (*P*=0.027, Figure S10.2F). Neither the RB1/P16 H-scores nor the different gene mutations (*TP53/RB1/STK11/KEAP1*) had a prognostic value (Figure 10.5A-F).

LCNEC with carcinoid morphology

Similar to carcinoids, the presence of *TP53* mutations was rare in the LCNEC with carcinoid morphology (1/6, 17%) in comparison to the non-carcinoid morphology LCNEC cohort (66/73 and 90%, *P*=0.001, Figure 10.2). In total eight LCNEC with carcinoid morphology were analyzed for RB1 and P16 IHC expression and three cases were RB1 positive and P16 negative. One LCNEC had a mitotic count <10 but with Ki-67 in parts of the tumor above 25%. The other seven had numerous mitoses counted (five >30 and two ≥10 but <30). An association between carcinoid morphology and OS in LCNEC was not observed *(P*=0.64, Figure S10.1C).

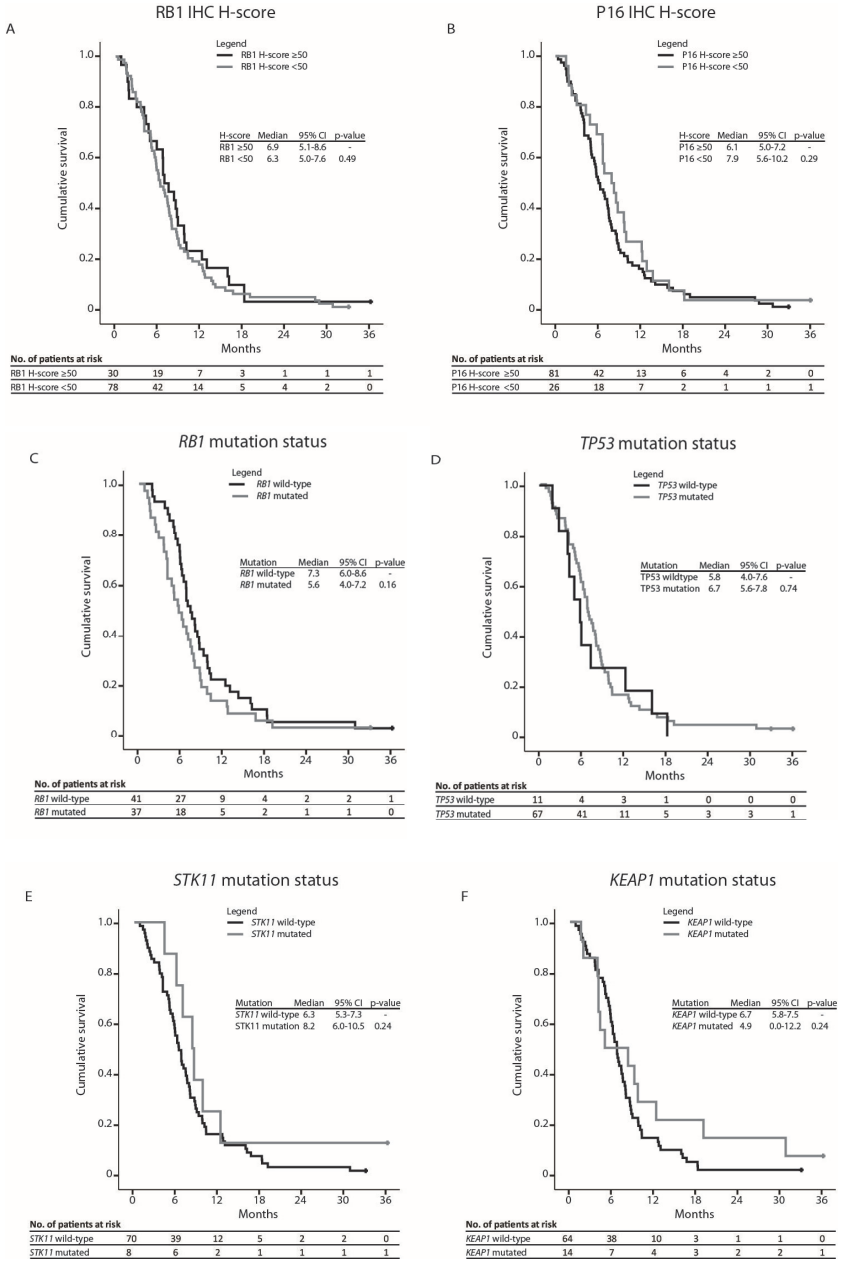

Figure 10.5 **A-B**) Overall survival in panel consensus LCNEC for RB1* (N=108) and P16* (N=107) IHC H-score. **C-F**) Overall survival for mutation status in consensus LCNEC (N=78)*
*Case 162 excluded for analyses
Abbreviations: No, number of; LCNEC, large cell neuroendocrine carcinoma; IHC, immunohistochemistry.

Discussion

Once diagnosed, LCNEC is frequently treated with SCLC-PE chemotherapy with poor responses[13,14,34,35]. Recently, we provided evidence that for LCNEC, NSCLC-GEM/TAX chemotherapy may be preferred over SCLC-PE chemotherapy[14]. Here we provide data suggesting that *RB1* wild-type LCNEC tumors respond better to NSCLC-GEM/TAX chemotherapy. However, no differences are observed for *RB1*-mutated LCNEC. Stratification of LCNEC into *RB1*-mutated and *RB1* wild-type subtypes, specifically in patients also expressing RB1 protein by IHC, may have implications for clinical management and requires prospective validation.

In line with what has been reported[17,18,36], we found *TP53* mutations in 85% of our pathology reviewed LCNEC cases, *RB1* in 47%, *STK11* in 10%, *KEAP1* in 18%, and *MEN1* in one tumor. *RB1* was co-altered with *TP53* in 92% of the *RB1*-mutated tumors, and mutations in *RB1* and *STK11* occurred in a mutually exclusive way. The frequency of *RB1* mutations identified in our study (47%) was slightly higher compared to the previous cohorts reported, but similar to another advanced disease LCNEC cohort (44%), suggesting that *RB1* is related to metastatic disease in LCNEC (±30 *versus* 45%)[17,18,36,37]. The frequency of *STK11* mutation was lower than expected, probably since we only covered 60% of the coding region of the gene *STK11*.

RB1 protein expression was completely lost in almost all of the *RB1*-mutated LCNEC, but also in 47% of the wild-type cases, likely due to gene inactivation by alternate mechanisms than mutation[18]. In total, RB1 protein expression was highly down-regulated or completely lost in 72% of the LCNEC analyzed. Similar results have been reported in a study on 78 LCNEC tumors, in which loss of RB1 protein was detected in 74% of the cases, while only 22% of them harbored an *RB1* mutation[36]. In a recent study on 24 *RB1* wild-type LCNEC tumors, the authors reported a retained expression of RB1 in all of them, possibly because the majority of tumors analyzed did not have metastatic disease[17]. Protein expression for RB1 is observed more frequently in LCNEC (33-55%) than in SCLC (≈90%) but clinical relevance has not been established[17,18,36,38,39] except for a recent prospective study reporting that patients with *RB1* wild-type SCLC, showed inferior OS and PFS when treated with SCLC-PE chemotherapy[40].

P16 and RB1 statuses are strongly correlated. P16 (*CDKN2A*) functions as an inhibitor of cyclin D-dependent kinases (CDK4/6) that phosphorylate *RB1* enabling cell proliferation[41]. *CDKN2A* (P16) and *RB1* inactivation seems to be mutually exclusive in LCNEC[18,42]. Hence, RB1 and P16 protein expression may have clinical value for diagnostic

and treatment purposes. Here we show that combined RB1 and P16 expression may be predictive for chemotherapy treatment. Furthermore, these markers can be used in the differential diagnosis of LCNEC *versus* SCLC as combined loss of RB1 protein and high P16 protein expression, is observed in 45-78% of LCNEC but almost always seen in SCLC (>90%)[36,38,39,41]. Hence, *RB1* and *P16* gene status and proteins expression patterns may have clinical value for diagnostic and treatment purposes in high-grade neuroendocrine carcinomas.

Recently a carcinoid subtype of LCNEC has been suggested[17,43]. We identified few LCNEC (12/148, 8%) with carcinoid morphology but with characteristic of a high-grade neuroendocrine carcinoma[17,43]. None of these LCNEC analyzed by NGS had a *MEN1* mutation, a gene that is frequently mutated in pulmonary carcinoid[44-46]. The relevance of LCNEC with carcinoid morphology is unclear and requires further evaluation. Nevertheless, the rare occurrence of *TP53* mutations in these subtypes suggests the existence of different LCNEC subtype.

This is the largest (population-based) study evaluating chemotherapy outcome related to mutational patterns in panel-reviewed LCNEC and the results we present here are of great interest for the clinical management of LCNEC. However, our study has few limitations due to its retrospective design. These data need to be confirmed in a prospective randomized clinical trial that stratifies LCNEC based on genomic subtypes and by RB1 and P16 protein expression and investigates outcome to NSCLC-GEM/TAX and SCLC-PE subtypes of chemotherapy.

In conclusion, we have shown that *RB1* mutation status and RB1/P16 protein expression are predictive markers for chemotherapy and may aid to guide therapeutic decisions in advanced LCNEC. Also, these markers can be applied in biopsy specimen of high-grade neuroendocrine carcinomas that cannot be separated into LCNEC or SCLC because of a lack of clear cytological features.

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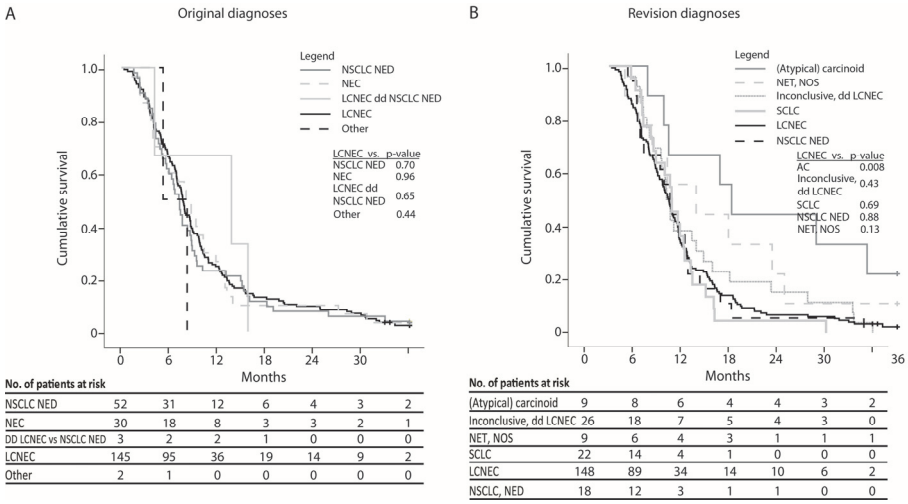
Supplemental material



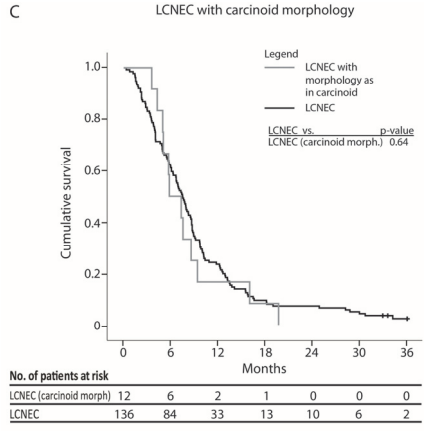


Figure S10.1 **A**) OS according to the original established diagnosis in patients included in the panel consensus revision (n=232). **B**) OS according to the panel consensus diagnoses, patients with carcinoids have longer OS. **C**) Comparison of consensus LCNEC diagnoses versus consensus LCNEC with carcinoid morphology, no differences were observed in prognosis

Abbreviations: No, number of; LCNEC, Large cell neuroendocrine carcinoma; NSCLC NED, non-small cell lung carcinoma with neuroendocrine differentiation; NEC, neuroendocrine carcinoma; dd, differential diagnosis; NET NOS, neuroendocrine tumor not otherwise specified; SCLC, small cell lung carcinoma; OS, overall survival

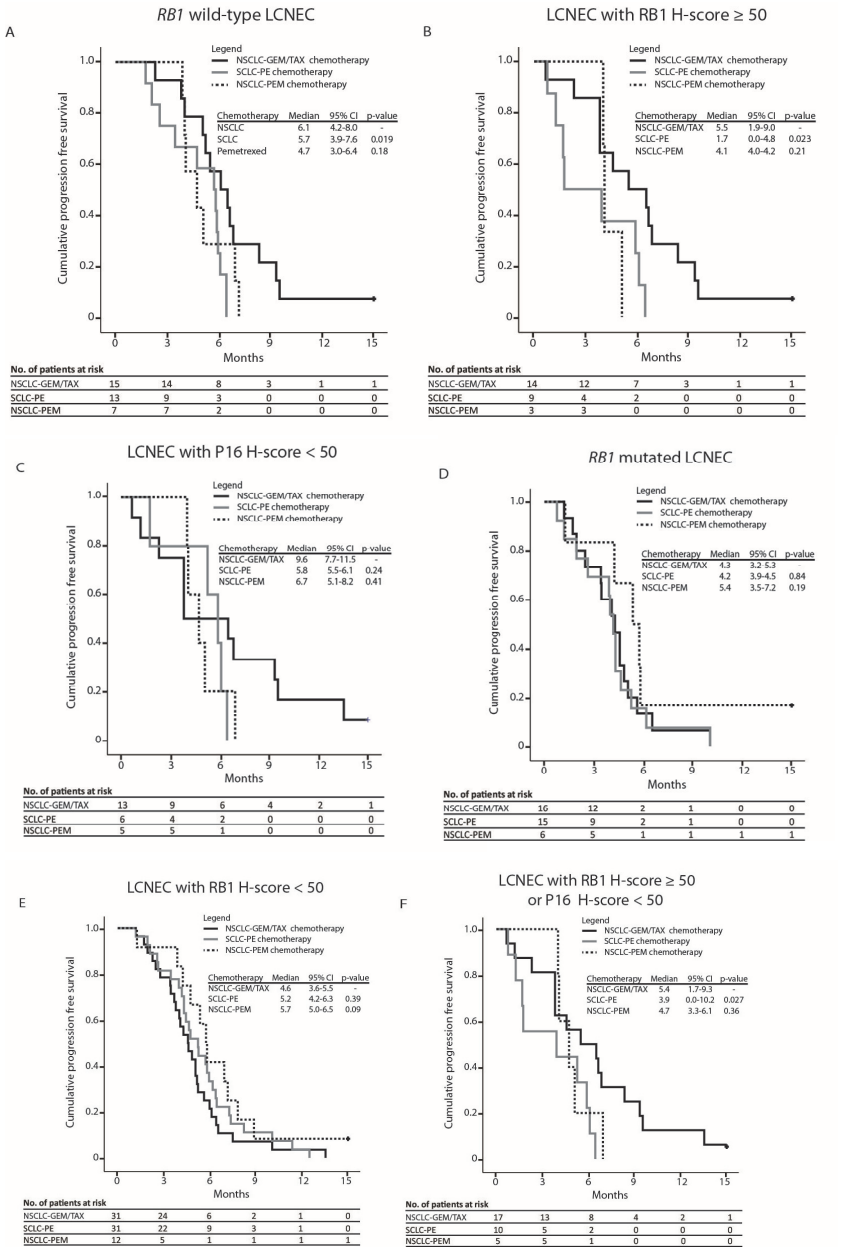


Figure S10.2 Progression free survival for subtypes of chemotherapy in consensus LCNEC in **A**) with *RB1* wild-type*; **B**) with an H-score ≥50 for RB1* IHC; **C**) with an H-score <50 for P16 IHC; **D**) with a *RB1* mutation; **E**) with an H-score <50 for RB1 IHC; **F**) with an H-score ≥50 for RB1* or <50 for P16 on IHC analysis

Abbreviations: LCNEC, Large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; NGS, next generation sequencing; No, number of. *Case 162 excluded from analyses

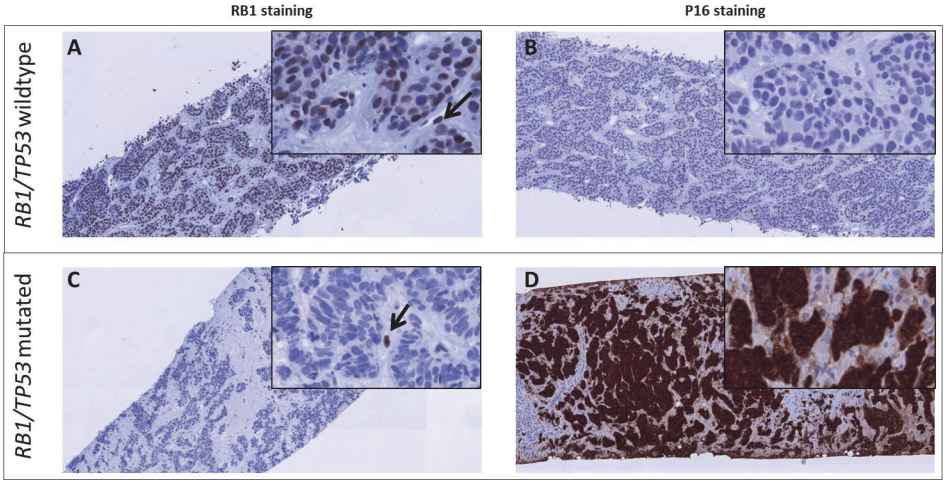


Figure S10.3 Original magnification overview x10 and insert x40 in consensus diagnosed LCNEC. **A**) Nuclear staining is observed on overview (10x) for RB1 protein staining (13A10) in LCNEC with *RB1* wild-type and *TP53* wild-type genes, the arrow indicates a positive internal control. **B**) in the identical case as A, staining for P16 protein (J8C) is negative. **C**) No nuclear staining for RB1 is observed in LCNEC mutated for *RB1* and *TP53* genes. **D**) However, this case shows strong nuclear and cytoplasmic staining for P16

Methods

Amplicon design and target enrichment

One hundred sixty-nine amplicons of 150 base pairs (bp) in size were designed covering the exons of TP53 (100%), RB1 (95%), STK11 (81%), KEAP1 (95%) and MEN1 (90%) with the Qiagen GeneRead DNAseq Custom V2 Builder tool reference CNGHS-02445X-169 (GRCh37). A validated in-house protocol (IARC) was used to perform multiplex PCR with four separate primer pools. Per pool, 5 µl of DNA diluted to a maximum of 4 ng/ul (0.60-4.0) were dispensed and air-dried. Subsequently 5 µl of the PCR mix were added (containing 2.5 µl primer, 1µl PCR mix, 0.34 µl HotStar Taq and 1.16 µl H_20) and the DNA was amplified in a 384 well plate as following: 15 min at 95°C, and 25 cycles of 15s at 95°C and 4 min at 60°C, and 10 min at 72°C. After amplification, the PCR products were pooled into a single reaction per sample.

Library preparation and next generation sequencing

The amplified PCR products were purified using NucleoMag® NGS Clean-up and Size Select beads (Macherey-Nagel, Germany). Purified PCR products were quantified by

Qubit DNA high-sensitivity assay kit (Invitrogen Corporation). A minimum of 100 ng of purified PCR product were included for library preparation with the NEBNext Fast DNA Library Prep Set (New England BioLabs, USA) following an in-house validated protocol (IARC). End-repair was performed and ligated to specific adapters and in-house prepared individual barcodes (Eurofins MWG Operon, Germany). Bead purification was applied to clean libraries and amplification was performed. Pooling of libraries was performed equimolar and loaded on a 2% agarose gel for electrophoresis (220V, 40 minutes). Using the GeneClean™ Turbo kit (MP Biomedicals, USA) pooled DNA library was recovered from selected fragments of 110-220 bp in length. Library quality and quantity was assessed on the Agilent 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies, USA). Sequencing of the libraries was performed on the Ion Torrent™ Proton Sequencer (Life Technologies Corp., USA) aiming for deep coverage (minimal 250×), using the Ion PI™ Hi-Q™OT2 200 Kit and the Ion PI™ Hi-Q™ sequencing 200 Kit with the Ion PI chip V3 (Life Technologies Corp., USA), following the manufacture's protocols.

RB1 and P16 immunohistochemistry staining

The N-terminal and C-terminal of the RB1 protein were targeted with antibody 4H1 (1:100, Cell Signaling Technology, USA) and 13A10 (1:100, Leica Biosystems, Germany). Additionally, P16 was targeted with antibody JC8 (1:400, Santa Cruz). Three-µm thick FFPE slides were stained using a Dako Autostainer Link 48 system with the EnVision FLEX™ visualization Kit (DAKO, Agilent, USA) according to standard protocols. For 13A10 and JC8 pH high antigen retrieval was used, and for 4H1 low pH antigen retrieval was used.

Table S10.1 Panel consensus review of possible LCNEC cases

Case	Consensus diagnosis	Unanimous diagnosis	Specimen	Mitosis	Necrosis	MIB	High-grade	Neuroendocrine morphology	CD56	Chr-A	Syn	TTF1	P63
5	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	++	+	+	+	-
10	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+++	-	-	+	UA.
14	LCNEC	2x LCNEC, 1x NSCLC NED	Resection	>30	Abundant	UA.	+	-	++	+++	++	+	UA.
19	LCNEC	Yes	EBB/TBB	>10 tot ≤30	None	>25	+	+	++	+	++	+	-
79	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+	+	UA.	+	UA.
119	LCNEC	2x LCNEC, 1x NSCLC NED	EBB/TBB	UA.	Abundant	UA.	+	UA.	+	UA.	+++	-	UA.
131	LCNEC	2X LCNEC, 1X SCLC	Resection	>30	Abundant	>25	+	+	+	-	+	+	-
2001	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	+++	UA.	+++	UA.	UA.
160	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>10 tot ≤30	None	>25	+	-	++	++	UA.	+	UA.
175	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	++	++	++	+	UA.
184	LCNEC	Yes	Resection	>30	Abundant	>25	+	+	+++	+	++	+	UA.
187	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>30	None	>25	+	+	+++	+	++	+	UA.
206	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+++	++	-	+	UA.
242	LCNEC	Yes	Needle	>30	None	>25	+	+	++	+	+	+	UA.
253	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+++	UA.	++	+	UA.
260	LCNEC	2X LCNEC, 1X COMBINED LCNEC SQCC	Needle	>10 tot ≤30	None	UA.	+	+	++	+	++	+	+
286	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	++	++	+++	+	-
263	LCNEC	Yes	Needle	>30	None	UA.	+	+	+++	++	++	+	-
348	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+++	++	+++	+	-
2006	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+++	UA.	++	+	-
368	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+++	UA.	UA.	-	UA.
369	LCNEC	2x LCNEC, 1x NSCLC NED	Resection	>30	Abundant	UA.	+	-	+++	+++	+++	+	-
377	LCNEC	Yes	Resection	>30	Abundant	>25	+	+	+++	-	+	-	-
132	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+	UA.	++	+	UA.
205	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+++	+	++	+	UA.
66	LCNEC	Yes	Needle	>10 tot ≤30	None	>25	+	+	++	+	+	+	UA.
173	LCNEC	2X LCNEC, 1X SCLC	Resection	>30	Abundant	>25	+	+	+	++	+	-	UA.
305	LCNEC	Yes	Needle	>30	None	UA.	+	+	++	-	UA.	UA.	UA.
268	LCNEC	Yes	Needle	>30	None	UA.	+	+	++	+	+++	UA.	UA.
180	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+	++	+++	-	-
128	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+	-	+	-	-
271	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+	++	++	-	UA.
315	LCNEC	Yes	Needle	>30	Dotlike	UA.	+	+	++	UA.	+++	+	-
347	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+	++	+++	+	-
2009	LCNEC	2x LCNEC, 1x LCNEC vs NSCLC NED	Resection	>30	None	UA.	+	+	+++	++	++	-	UA.
32	LCNEC (carcinoid morphology)	Yes	Resection	>10 tot ≤30	Dotlike	>25	+	Carc+	++	++	+	+	-
292	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+++	-	+++	+	-
56	LCNEC	Yes	Needle	UA.	Abundant	>25	+	-	+++	+	+++	+	UA.
13	LCNEC	Yes	Needle	>10 tot ≤30	None	>25	+	+	+++	++	++	+	-
18	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+	++	+	+	UA.
123	LCNEC	2X LCNEC, 1X SCLC	EBB/TBB	>30	Abundant	>25	+	+	+++	++	++	+	UA.

Table S10.1 (continued)

Case	Consensus diagnosis	Unanimous diagnosis	Specimen	Mitosis	Necrosis	MIB	High-grade	Neuroendocrine morphology	CD56	Chr-A	Syn	TTF1	P63
125	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+	-	+	-	UA.
157	LCNEC	Yes	Needle	>30	Abundant	>25	+	+	+	+	UA.	+	UA.
174	LCNEC	Yes	EBB/TBB	>30	None	UA.	+	+	+++	-	+++	+	-
207	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	-	++	+++	+	UA.
222	LCNEC	Yes	Needle	>30	Abundant	>25	+	+	++	++	+	+	-
327	LCNEC	2x LCNEC, 1x SCLC dd LCNEC	Needle	>30	Abundant	>25	+	+	+	UA.	-	-	UA.
297	LCNEC	2X LCNEC, 1X COMBINED LCNEC-SCLC	Needle	>30	None	UA.	+	+	-	-	+	+	UA.
341	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	++	+	+	+	-
372	LCNEC (carcinoid morphology)	Yes	Needle	>30	Dotlike	>25	+	Carc+	+++	UA.	+++	+	UA.
380	LCNEC	Yes	EBB/TBB	>30	Abundant	UA.	+	+	+++	-	+	-	-
381	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	++	+	UA.	-	-
2002	LCNEC (carcinoid morphology)	Yes	Resection	>30	Dotlike	UA.	+	Carc+	+++	++	+++	+	UA.
61	LCNEC	Yes	EBB/TBB	>10 tot ≤30	None	>25	+	+	++	UA.	UA.	UA.	UA.
144	LCNEC	Yes	EBB/TBB	>10 tot ≤30	None	UA.	+	+	++	UA.	+++	+	UA.
393	LCNEC	Yes	Resection	>10 tot ≤30	Abundant	UA.	+	+	+	++	+++	UA.	UA.
188	LCNEC	Yes	Needle	>10 tot ≤30	None	>25	+	+	+++	+++	+	+	UA.
117	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	+	-	+	+	UA.
176	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	-	+++	++	+	UA.
314	LCNEC (carcinoid morphology)	Yes	Resection	>30	Dotlike	>25	+	Carc+	-	+++	+++	-	UA.
229	LCNEC	Yes	EBB/TBB	UA.	Abundant	>25	+	+	-	+	++	+	-
357	LCNEC	Yes	Biopsy NOS	>30	Abundant	>25	+	+	+++	++	++	UA.	UA.
367	LCNEC	Yes	EBB/TBB	>10 tot ≤30	None	>25	+	+	UA.	++	+	+	-
8	LCNEC	Yes	EBB/TBB	>10 tot ≤30	Abundant	UA.	+	+	UA.	++	++	UA.	UA.
60	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	++	-	+++	-	UA.
130	LCNEC	2X LCNEC, 1X SCLC	Needle	>30	Abundant	UA.	+	+	++	+	+	-	UA.
169	LCNEC	2x LCNEC, 1x NSCLC NED	EBB/TBB	>30	Abundant	UA.	+	-	++	-	-	UA.	UA.
191	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>30	None	UA.	+	-	++	+	UA.	-	UA.
221	LCNEC	2X LCNEC, 1X SCLC	Needle	>10 tot ≤30	Abundant	>25	+	+	+++	-	++	-	-
257	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	UA.	+++	+++	-	UA.
261	LCNEC	Yes	Resection	>30	Abundant	UA.	+	+	-	-	+	-	-
265	LCNEC	Yes	Resection	>30	Abundant	>25	+	+	+	-	++	+	-
316	LCNEC	Yes	Needle	>30	None	UA.	+	+	++	UA.	+++	+	-
353	LCNEC	Yes	Needle	>30	None	UA.	+	+	UA.	UA.	++	-	-
2010	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+++	+	-	+	UA.
80	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	>25 and ≤25	+	+	+	UA.	+	+	-
162	LCNEC	Yes	EBB/TBB	>10 tot ≤30	None	>25	+	+	+++	+++	+	UA.	UA.
275	LCNEC (carcinoid morphology)	Yes	Resection	>30	Abundant	UA.	+	Carc+	++	++	-	-	-
2004	LCNEC (too much necrosis for carcinoid)	2x LCNEC, 1x NSCLC NED / AC	Resection	<10	Abundant	heterogeneous >25 and <25	+/-	+	++	++	+++	+	UA.

Table S10.1 (continued)

Case	Consensus diagnosis	Unanimous diagnosis	Specimen	Mitosis	Necrosis	MIB	High-grade	Neuroendocrine morphology	CD56	Chr-A	Syn	TTF1	P63
6	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>10 tot ≤30	Abundant	UA.	+	-	+++	++	+++	+	UA.
59	LCNEC	2X LCNEC, 1X SCLC	Needle	>10 tot ≤30	Abundant	>25	+	+	+++	+	++	+	UA.
67	LCNEC	Yes	Needle	>10 tot ≤30	None	UA.	+	+	+	-	UA.	UA.	-
77	LCNEC (carcinoid morphology)	Yes	Needle	>30	Dotlike	UA.	+	Carc+	UA.	++	UA.	UA.	UA.
108	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>10 tot ≤30	Abundant	UA.	+	-	+++	++	+	+	UA.
139	LCNEC	Yes	Needle	>10 tot ≤30	None	UA.	+	+	++	-	+	+	UA.
170	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	+	++	+++	-	UA.
197	LCNEC	2x LCNEC, 1x SCLC dd LCNEC	Needle	>30	None	>25	+	+	+++	UA.	UA.	+	-
198	LCNEC	Yes	Needle	>30	None	UA.	+	+	++	++	++	+	UA.
199	LCNEC	Yes	EBB/TBB	>10 tot ≤30	None	UA.	+	+	++	+	+++	+	-
200	LCNEC	Yes	Needle	>30	None	>25	+	+	+	+	++	-	UA.
220	LCNEC	Yes	EBB/TBB	>30	Dotlike	>25	+	+	-	++	+++	+	UA.
249	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	++	++	+	+	UA.
254	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	-	++	++	+	UA.
280	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	-	-	++	-	UA.
288	LCNEC	Yes	Needle	>10 tot ≤30	None	>25	+	+	+++	UA.	+++	+	UA.
311	LCNEC	Yes	EBB/TBB	>10 tot ≤30	None	>25	+	+	++	++	+++	+	UA.
312	LCNEC	Yes	Needle	>10 tot ≤30	None	UA.	+	+	+++	++	+++	+	UA.
329	LCNEC	Yes	EBB/TBB	>30	Abundant	>25	+	+	+	++	++	-	-
356	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	++	++	+++	+	UA.
360	LCNEC (carcinoid morphology)	Yes	Needle	>10 tot ≤30	None	>25	+	Carc+	+++	++	+++	+	-
382	LCNEC	2x LCNEC, 1x SCLC dd LCNEC	Needle	>30	Abundant	>25	+	+	+	+++	+	-	-
2005	LCNEC	2x LCNEC, 1x LCNEC vs. SCLC	Resection	>30	Abundant	UA.	+	+	++	++	++	+	UA.
159	LCNEC	2x LCNEC, 1x NSCLC NED	EBB/TBB	UA.	Abundant	>25	+	UA.	+	++	++	UA.	UA.
53	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>10 tot ≤30	None	UA.	+	-	++	UA.	UA.	UA.	UA.
51	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>30	Abundant	UA.	+	-	-	UA.	++	-	UA.
216	LCNEC	Yes	Needle	≤10	Abundant	UA.	+	+	++	UA.	UA.	-	-
252	LCNEC	2x LCNEC, 1x combined LCNEC	Needle	>30	Abundant	UA.	+	+	+	++	++	-	-
300	LCNEC	2x LCNEC, 1x NSCLC NED	EBB/TBB	>10 tot ≤30	Abundant	>25	+	-	+++	+	++	+	-
27	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	UA.	-	+	+	UA.
4	SCLC	Yes	Resection	>10 tot ≤30	Abundant	>25	+	+	+	++	+++	+	UA.
15	Heterogeneous, LCNEC VS SCLC	1x high-grade NEC, 1x NET dd SCLC, 1x SCLC dd LCNEC	Needle	UA.	None	UA.	UA.	UA.	+++	+	+	UA.	UA.
17	NSCLC NED	2x NSCLC NED, 1x LCNEC	Needle	>10 tot ≤30	Abundant	UA.	+	-	+++	++	++	+	-
25	Heterogeneous, LCNEC VS SCLC	2x LCNEC dd SCLC, 1x SCLC	Resection	UA.	Abundant	UA.	+	UA.	+	+	+	+	UA.
26	NSCLC NED	2x NSCLC NED, 1x LCNEC	EBB/TBB	>10 tot ≤30	None	UA.	+	-	++	+++	+	+	UA.
28	NSCLC NED	Yes	Needle	UA.	None	UA.	UA.	-	UA.	++	-	+	UA.
29	LCNEC	Yes	Needle	>10 tot ≤30	None	UA.	+	+	+++	UA.	UA.	+	UA.
34	NET NOS	Yes	EBB/TBB	UA.	None	UA.	UA.	UA.	-	+	+	+	UA.
37	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	≤10	Abundant	UA.	+	UA.	+++	++	+++	UA.	-

Table S10.1 (continued)

Case	Consensus diagnosis	Unanimous diagnosis	Specimen	Mitosis	Necrosis	MIB	High-grade	Neuroendocrine morphology	CD56	Chr-A	Syn	TTF1	P63
40	LCNEC	Yes	Needle	>10 tot ≤30	None	UA.	+	+	+	UA.	UA.	+	UA.
43	NSCLC NED	2x NSCLC NED, 1x LCNEC	Needle	UA.	Abundant	UA.	+	UA.	+++	-	UA.	+	UA.
46	SCLC	Yes	Needle	>30	Abundant	>25	+	+	+++	UA.	UA.	+	UA.
50	NSCLC NED	2x NSCLC NED, 1x NSCLC NOS	Resection	>10 tot ≤30	Abundant	UA.	+	-	-	-	+	-	UA.
52	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x NSCLC NED, 1x LCNEC, 1x dd LCNEC or SCLC	Needle	>30	None	UA.	+	+	++	+	+	+	+
54	NSCLC NED	2x NSCLC NED, 1x LCNEC	Needle	>10 tot ≤30	None	UA.	+	-	-	-	++	+	UA.
57	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x NSCLC NED, 1x LCNEC, 1x dd LCNEC or SCLC	Resection	>30	Abundant	>25	+	-	+	-	+	-	UA.
58	NET NOS	Yes	EBB/TBB	≤10	None	UA.	-	+	+++	UA.	UA.	+	UA.
72	SCLC	Yes	Needle	>30	None	UA.	+	-	+++	UA.	UA.	+	UA.
74	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	++	+++	+++	+	UA.
78	NSCLC NED	Yes	EBB/TBB	>10 tot ≤30	None	UA.	+	-	+++	+	UA.	+	UA.
81	SCLC	Yes	EBB/TBB	UA.	None	UA.	UA.	-	+	UA.	UA.	+	UA.
91	Heterogeneous, LCNEC VS SCLC	1x LCNEC, 1x SCLC, 1x LCNEC or SCLC	Resection	>30	None	UA.	+	-	+	UA.	-	UA.	UA.
95	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	++	-	++	+	UA.
100	NSCLC NED	2x NSCLC NED, 1x LCNEC	Resection	>10 tot ≤30	None	UA.	+	-	++	UA.	UA.	+	UA.
105	LCNEC (carcinoid morphology)	Yes	Needle	>30	Abundant	UA.	+	Carc+	+++	UA.	UA.	-	UA.
106	SCLC	Yes	Resection	>30	Abundant	UA.	+	-	+	-	-	+	UA.
109	NET NOS	Yes	Needle	UA.	None	UA.	UA.	UA.	+++	+++	++	-	UA.
110	Carcinoid	Yes	Needle	≤10	None	UA.	-	+	-	-	+	-	UA.
111	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x NSCLC NED, 1x SCLC, 1x NET dd SCLC or LCNEC	EBB/TBB	>30	None	UA.	+	UA.	+	+	++	-	UA.
121	Heterogeneous, NSCLC NED VS LCNEC	1x NSCLC NED, 1x LCNEC, 1x high-grade NEC	EBB/TBB	UA.	None	UA.	UA.	UA.	+++	UA.	+	+	UA.
124	LCNEC	2X LCNEC, 1X SCLC	EBB/TBB	>30	Dotlike	UA.	+	+	+++	UA.	UA.	-	UA.
127	NSCLC NED	2x NSCLC NED, 1x LCNEC	EBB/TBB	>10 tot ≤30	None	UA.	+	-	+	UA.	UA.	UA.	UA.
129	SCLC	Yes	Needle	>10 tot ≤30	None	UA.	+	+	+++	+	++	+	UA.
140	SCLC	Yes	EBB/TBB	UA.	None	>25	+	UA.	++	++	+	UA.	UA.
141	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>10 tot ≤30	None	UA.	+	-	+	-	+	+	UA.
148	NET NOS	Yes	EBB/TBB	UA.	Abundant	UA.	+	UA.	++	UA.	UA.	+	UA.
151	SCLC	Yes	Resection	>30	Abundant	UA.	+	+	+++	++	-	+	UA.
152	NSCLC NED	2x NSCLC NED, 1x LCNEC	EBB/TBB	>30	Abundant	UA.	+	-	+	UA.	UA.	+	-
154	LCNEC	Yes	EBB/TBB	>30	Dotlike	UA.	+	+	+	-	+	+	UA.
155	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	>25	+	+	++	+	UA.	-	UA.
156	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x SCLC, 1x NSCLC NED, 1x LCNEC	EBB/TBB	UA.	None	UA.	UA.	UA.	UA.	+	+	UA.	UA.
161	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	UA.	Abundant	UA.	+	UA.	++	+	+	+	UA.
163	SCLC	Yes	Resection	>30	Dotlike	>25	+	+	++	+	++	+	UA.
165	Heterogeneous, LCNEC VS SCLC	2x NSCLC NED vs SCLC, 1x LCNEC	EBB/TBB	>10 tot ≤30	None	>25	+	UA.	++	++	UA.	-	UA.

Table S10.1 (continued)

Case	Consensus diagnosis	Unanimous diagnosis	Specimen	Mitosis	Necrosis	MIB	High-grade	Neuroendocrine morphology	CD56	Chr-A	Syn	TTF1	P63
166	LCNEC (carcinoid morphology)	Yes	Needle	>10 tot ≤30	Abundant	>25	+	Carc+	+	+++	+++	+	-
171	SCLC	Yes	EBB/TBB	UA.	Abundant	>25	+	+	++	-	-	-	UA.
172	NSCLC NED	2x NSCLC NED, 1x LCNEC	EBB/TBB	>10 tot ≤30	None	UA.	+	UA.	++	++	++	+	UA.
177	NSCLC NED	2x NSCLC NED, 1x LCNEC	EBB/TBB	UA.	None	UA.	UA.	UA.	+++	UA.	+++	UA.	UA.
182	NET NOS	Yes	Resection	>10 tot ≤30	None	≤25	+	+	+	-	-	-	+
183	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	>10 tot ≤30	Abundant	UA.	+	-	-	++	++	-	UA.
185	Heterogeneous, NSCLC NED VS LCNEC	1x NSCLC NED, 1x LCNEC, 1x NSCLC NED dd LCNEC	EBB/TBB	UA.	None	UA.	UA.	UA.	+	++	UA.	-	UA.
186	LCNEC	2x LCNEC, 1x NET NOS	EBB/TBB	UA.	Abundant	UA.	+	UA.	+	++	++	+	UA.
189	NSCLC NED	2x NSCLC NED, 1x LCNEC	EBB/TBB	≤10	Abundant	>25	+	-	++	-	-	-	UA.
190	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x LCNEC, 1x NSCLC NED, 1x SCLC or NSCLC NED	EBB/TBB	>10 tot ≤30	None	>25	+	-	+++	UA.	UA.	-	-
193	LCNEC	Yes	Needle	>30	None	>25	+	+	++	++	++	+	UA.
196	Heterogeneous, NSCLC NED VS LCNEC	1x NSCLC NED, 1x LCNEC, 1x NET NOS	EBB/TBB	UA.	Abundant	UA.	+	-	++	UA.	UA.	+	UA.
201	NET NOS	Yes	Needle	UA.	None	UA.	UA.	+	+	UA.	+	+	UA.
204	LCNEC	Yes	Needle	≤10	Abundant	UA.	+	+	++	UA.	UA.	+	UA.
209	LCNEC	2x LCNEC, 1x NSCLC NED	EBB/TBB	>10 tot ≤30	None	UA.	+	+	++	+++	+++	+	-
214	Carcinoid	2x Carcinoid, 1x LCNEC vs carcinoid	Needle	≤10	None	UA.	-	+	++	+++	++	+	-
215	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x SCLC, 1x NSCLC NED, 1x LCNEC	Resection	>30	Abundant	>25	+	+	+++	++	+++	-	-
219	Carcinoid	Yes	EBB/TBB	UA.	None	UA.	UA.	+	+++	UA.	+++	-	-
225	LCNEC	Yes	Needle	≤10	Abundant	UA.	+	+	-	++	+	-	-
230	SCLC	Yes	Needle	>30	Abundant	UA.	+	+	+++	++	-	+	UA.
231	Carcinoid	Yes	Needle	≤10	None	≤25	-	+	UA.	+++	+	-	-
232	LCNEC	Yes	EBB/TBB	>30	Abundant	>25	+	+	+	-	+++	+	UA.
233	LCNEC	2x LCNEC, 1x NET NOS	EBB/TBB	≤10	None	UA.	-	+	UA.	++	++	+	-
234	SCLC	Yes	Needle	>30	None	>25	+	+	+++	++	+++	+	-
235	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	≤10	None	UA.	-	+	+++	++	+++	+	UA.
236	LCNEC	Yes	Needle	>10 tot ≤30	Dotlike	>25	+	+	+	++	++	+	UA.
239	SCLC	2x DD SCLC, 1x NET NOS	EBB/TBB	UA.	Abundant	UA.	+	UA.	++	++	++	-	-
241	SCLC	Yes	Needle	>10 tot ≤30	Dotlike	UA.	+	-	+++	++	+++	-	UA.
251	NSCLC NED	2x NSCLC NED, 1x LCNEC	Needle	>10 tot ≤30	Abundant	UA.	+	-	+++	++	+	UA.	UA.
255	Carcinoid	Yes	Needle	≤10	None	UA.	-	+	+	+++	UA.	-	UA.
256	SCLC	2x SCLC, 1x LCNEC vs. SCLC	EBB/TBB	UA.	Abundant	>25	+	UA.	++	++	+++	-	UA.
258	Heterogeneous, LCNEC VS SCLC	1x LCNEC, 1x SCLC, 1x LCNEC or SCLC	Needle	>10 tot ≤30	Dotlike	UA.	+	+	+++	++	+++	+	UA.
259	LCNEC	Yes	Needle	>30	Dotlike	>25	+	+	+	+++	++	+	UA.
262	NSCLC NED	2x NSCLC NED, 1x LCNEC	Needle	>10 tot ≤30	None	>25	+	UA.	+++	++	++	-	UA.
264	LCNEC	2x LCNEC, 1x NSCLC NED	EBB/TBB	>10 tot ≤30	Abundant	UA.	+	-	-	+	++	+	UA.
267	LCNEC	2x LCNEC, 1x SCLC dd LCNEC	Needle	>30	Abundant	UA.	+	+	+++	-	UA.	+	UA.
270	LCNEC	2x LCNEC, 1x NSCLC NED	Needle	≤10	Dotlike	>25	+	+	+++	+++	UA.	+	UA.

Table S10.1 (continued)

Case	Consensus diagnosis	Unanimous diagnosis	Specimen	Mitosis	Necrosis	MIB	High-grade	Neuroendocrine morphology	CD56	Chr-A	Syn	TTF1	P63
272	Heterogeneous, NSCLC NED VS LCNEC	1x NSCLC NED, 1x LCNEC, 1x NSCLC NED or LCNEC	EBB/TBB	>30	None	UA.	+	-	+++	UA.	UA.	-	-
279	Carcinoid	Yes	Needle	≤10	None	UA.	-	+	+++	+++	+++	UA.	UA.
285	NSCLC NED	2x NSCLC NED, 1x LCNEC	Needle	>10 tot ≤30	Abundant	UA.	+	-	-	UA.	++	+	UA.
287	Heterogeneous, NSCLC NED VS LCNEC	1x NSCLC NED, 1x LCNEC, 1x NSCLC NED or LCNEC	EBB/TBB	UA.	Dotlike	UA.	+/-	-	UA.	++	UA.	-	-
293	LCNEC	2X LCNEC, 1X COMBINED LCNEC-SCLC	Needle	>30	Abundant	UA.	+	+	+++	+	++	+	UA.
295	Carcinoid	Yes	Needle	≤10	None	≤25	-	+	++	+++	+++	+	-
296	LCNEC	Yes	Needle	UA.	None	>25	+	+	+++	+	+++	+	UA.
298	LCNEC	2X LCNEC, 1X SCLC	EBB/TBB	UA.	Abundant	UA.	+	+	++	-	+++	UA.	-
299	LCNEC	2x LCNEC, 1x SCLC dd LCNEC	Needle	UA.	Abundant	UA.	+	+	+++	+++	++	+	UA.
301	LCNEC	Yes	Needle	>30	Dotlike	>25	+	+	+	+++	++	+	-
308	LCNEC (carcinoid morphology)	Yes	Needle	>10 tot ≤30	None	UA.	+	Carc+	+	UA.	UA.	+	-
309	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x LCNEC, 1x NSCLC NED, 1x SCLC or LCNEC	Resection	>30	Abundant	UA.	+	-	++	+++	+++	+	-
313	LCNEC	Yes	Needle	>30	Abundant	>25	+	+	+++	+	+++	+	UA.
317	SCLC	Yes	Resection	>30	Abundant	UA.	+	+	+++	++	+++	+	UA.
322	LCNEC	Yes	Needle	>10 tot ≤30	Abundant	UA.	+	+	-	++	++	+	UA.
323	LCNEC	2x LCNEC, 1x SCLC dd LCNEC	Needle	>30	None	>25	+	+	++	-	-	-	UA.
324	Heterogeneous, LCNEC VS SCLC	2x LCNEC dd SCLC, 1x SCLC	Needle	≤10	None	>25	+	+	+++	++	+	+	UA.
325	Carcinoid	Yes	EBB/TBB	≤10	None	>25	+	+	UA.	+++	+	+	UA.
326	Heterogeneous, LCNEC VS SCLC	1x SCLC / LCNEC, 1x LCNEC dd SCLC, 1x SCLC	Resection	>30	Abundant	>25	+	+	+	++	++	+	-
330	SCLC	2x DD SCLC, 1x not evaluable	EBB/TBB	UA.	None	UA.	UA.	UA.	++	UA.	UA.	UA.	-
331	Heterogeneous, LCNEC VS SCLC	Yes	Needle	>30	Abundant	UA.	+	+	+++	++	++	+	-
334	Heterogeneous, LCNEC VS SCLC	2x LCNEC dd SCLC, 1x SCLC	Needle	>30	None	>25	+	+	+++	++	+++	-	UA.
337	SCLC	Yes	Needle	UA.	None	>25	+	+	+++	+++	+++	UA.	UA.
338	SCLC	Yes	EBB/TBB	UA.	None	UA.	UA.	UA.	+++	+	+	+	-
342	Heterogeneous, LCNEC VS SCLC	1x SCLC, 1x SCLC vs LCNEC, 1x LCNEC	EBB/TBB	UA.	Abundant	UA.	+	+	++	+	+	+	UA.
343	LCNEC	Yes	Needle	>30	None	>25	+	+	+	++	++	-	-
346	Heterogeneous, LCNEC VS SCLC	1x SCLC dd LCNEC, 1x NSCLC NED dd SCLC, 1x NET NOS	EBB/TBB	UA.	Dotlike	UA.	+/-	UA.	+++	-	-	-	UA.
349	LCNEC	Yes	Needle	>30	Abundant	UA.	+	+	+	+	UA.	+	-
352	Heterogeneous, NSCLC NED VS LCNEC	1x NSCLC NED, 1x LCNEC, 1x NSCLC NED or LCNEC	Needle	>30	Dotlike	>25	+	-	-	++	++	-	UA.
361	Carcinoid	Yes	Resection	≤10	Dotlike	≤25	-	+	+++	+++	+++	+	UA.
362	LCNEC (carcinoid morphology)	2x LCNEC (carcinoid morphology), 1x NSCLC NED	Needle	≤10	Abundant	>25	+	Carc+	++	+++	+++	+	-

Table S10.1 (continued)

Case	Consensus diagnosis	Unanimous diagnosis	Specimen	Mitosis	Necrosis	MIB	High-grade	Neuroendocrine morphology	CD56	Chr-A	Syn	TTF1	P63
363	SCLC	Yes	EBB/TBB	>30	None	>25	+	+	++	+	+	UA.	UA.
366	Heterogeneous, LCNEC VS SCLC	2x LCNEC dd SCLC, 1x LCNEC	EBB/TBB	>30	None	UA.	+	+	+	+++	+++	-	-
373	LCNEC	2x LCNEC, 1x NET NOS	EBB/TBB	UA.	None	UA.	UA.	UA.	+++	+++	++	-	-
374	NSCLC NED	2x NSCLC NOS, 1x dd SCLC	Needle	UA.	Abundant	>25	+	-	+	UA.	UA.	-	-
376	SCLC	Yes	Needle	>30	Abundant	UA.	+	+	+++	++	++	+	UA.
378	NET NOS	Yes	EBB/TBB	UA.	None	≤25	-	-	+++	++	+++	-	UA.
379	NET NOS	Yes	Needle	UA.	None	UA.	UA.	UA.	+++	++	+++	+	UA.
387	Heterogeneous, NSCLC NED VS LCNEC VS SCLC	1x LCNEC, 1x LCNEC or SCLC, 1x NSCLC NED	Resection	>30	Abundant	>25	+	-	+++	+	++	-	-
388	NET NOS	1x NSCLC NED combined with SqCC, 1x SCLC combined SqCC, 1x LCNEC combined SqCC	Needle	≤10	None	>25	+	-	++	+	++	+	UA.
2007	NSCLC NED	Yes	Biopsy NOS	UA.	Abundant	UA.	+	UA.	-	-	++	+	-
2003	SCLC	Yes	Needle	>30	Abundant	>25	+	+	++	++	++	-	UA.

Abbreviations: UA, unavailable; IHC, immunohistochemistry; NOS, not otherwise specified; NSCLC NED, NSCLC with neuroendocrine IHC differentiation; AC, atypical carcinoid; EBB, endobronchial biopsy; TBB, trans bronchial biopsy; Syn, synaptophysin; Chr-A, chromogranin-A; SCLC, small cell lung carcinoma; LCNEC, large cell neuroendocrine carcinoma; dd, differential diagnosis; NEM; neuroendocrine morphology without staining for neuroendocrine markers; vs, versus

Table S10.2 Data no performed next-generation sequencing analyses and protein expression analyses

Case	Coverage	% tumor	Tp53	RB1	STK11	KEAP1	Men1	Chemotherapy	H-score RB1	H-score RB1 4H1	H-score P16
5	2771,5	60	nonsynonymous SNV	stopgain	WT	WT	WT	SCLC type	0		300
10	1623	50	frameshift deletion	frameshift deletion	WT	WT	WT	NSCLC type	0	0	300
14	1475	60	nonsynonymous SNV	frameshift insertion	WT	WT	WT	SCLC type	0		300
19	2127,5	50	nonsynonymous SNV	frameshift deletion	WT	WT	WT	NSCLC type	0	0	300
79	719	80	nonsynonymous SNV	stopgain	WT	WT	WT	NSCLC type	0	0	300
119	4384	30	frameshift insertion	nonframeshift deletion	WT	WT	WT	SCLC type	0		250
131	1180,5	80	nonsynonymous SNV	stopgain	WT	WT	WT	NSCLC type	0	0	300
2001	3000	50	frameshift deletion	stopgain	WT	WT	WT	NSCLC type	0		300
160	2681	50	nonsynonymous SNV	NA	WT	WT	WT	NSCLC type	0		300
175	1659,5	70	stopgain	frameshift insertion	WT	WT	WT	NSCLC pemetrexed type	0	0	300
184	1271	80	nonsynonymous SNV	frameshift deletion	WT	WT	WT	NSCLC type	0	0	300
187	2290,5	40	nonsynonymous SNV	frameshift deletion	WT	WT	WT	NSCLC type	0		300
206	6317,5	40	frameshift insertion	NA	WT	WT	WT	NSCLC pemetrexed type	0		300
242	1321	70	nonsynonymous SNV	frameshift deletion	WT	WT	WT	SCLC type	0		300
253	890	70	nonsynonymous SNV	stopgain	WT	WT	WT	SCLC type	0	0	300
260	1623	80	nonsynonymous SNV	NA	WT	WT	WT	NSCLC pemetrexed type	0		300
286	3267	90	nonsynonymous SNV	NA	WT	WT	WT	SCLC type	0		300
263	3557	60	nonsynonymous SNV	stopgain	WT	WT	WT	SCLC type	0		0
348	5404	50	NA	NA	WT	WT	WT	SCLC type	0		270
2006	1243	90	frameshift deletion	stopgain	WT	WT	WT	NSCLC pemetrexed type	0	0	300
368	1663,5	90	frameshift deletion	stopgain	WT	WT	WT	NSCLC pemetrexed type	0	0	280
369	613,5	80	NA	frameshift deletion	WT	WT	WT	SCLC type	0	0	300
377	712,5	80	frameshift deletion	frameshift deletion	WT	WT	WT	NSCLC pemetrexed type	0	0	300
132	5331	40	nonsynonymous SNV	frameshift deletion	WT	WT	WT	NSCLC type	0		0
205	3663,5	30	frameshift deletion	frameshift deletion	WT	WT	WT	SCLC type	0	0	200
66	1355,5	90	nonsynonymous SNV	NA	WT	WT	WT	SCLC type	95		300
173	1160	80	nonsynonymous SNV	NA	WT	WT	WT	NSCLC type	200	150	300
305	6611	80	nonsynonymous SNV	nonsynonymous SNV	WT	WT	WT	SCLC type	100		300
268	6356	20	stopgain	nonsynonymous SNV	WT	nonsynonymous SNV	WT	NSCLC type	0		300
180	965	70	nonsynonymous SNV	frameshift insertion	WT	nonsynonymous SNV	WT	NSCLC type	0	0	300
128	1692	50	stopgain	frameshift deletion	WT	nonsynonymous SNV	WT	NSCLC type	0		300
271	1913,5	60	nonsynonymous SNV	stopgain	WT	nonsynonymous SNV	WT	NSCLC type	0		300
315	4927,5	80	nonsynonymous SNV	NA	WT	stopgain	WT	SCLC type	0		300

Table S10.2 (continued)

Case	Coverage	% tumor	Tp53	RB1	STK11	KEAP1	Men1	Chemotherapy	H-score RB1	H-score RB1 4H1	H-score P16
347	2897	70	nonsynonymous SNV	stopgain	WT	nonsynonymous SNV	WT	SCLC type	0		300
2009	789,5	90	WT	frameshift deletion	WT	WT	WT	NSCLC type	0	0	300
32	859,5	90	WT	frameshift deletion	WT	WT	WT	SCLC type	0	0	300
292	1782	80	WT	NA	WT	WT	WT	NSCLC type	0	0	300
56	6355	70	nonsynonymous SNV	WT	WT	WT	WT	NSCLC pemetrexed type	0		300
13	1090	80	nonsynonymous SNV	WT	WT	WT	WT	SCLC type	0		300
18	2487,5	50	nonsynonymous SNV	WT	WT	WT	WT	SCLC type	0		300
123	4043,5	70	nonsynonymous SNV	WT	WT	WT	WT	Chemotherapy unknown	0		280
125	2145,5	90	nonsynonymous SNV	WT	WT	WT	WT	Chemotherapy unknown	0	0	220
157	6827	60	stopgain	WT	WT	WT	WT	NSCLC type	0		300
174	2829	100	nonframeshift deletion	WT	WT	WT	WT	SCLC type	0		300
207	3128,5	70	nonsynonymous SNV	WT	WT	WT	WT	NSCLC pemetrexed type	0		200
222	4876	40	stopgain	WT	WT	WT	WT	Chemotherapy unknown	0		300
327	1166	80	nonsynonymous SNV	WT	WT	WT	WT	NSCLC type	0		160
297	2598,5	40	frameshift deletion	WT	WT	WT	WT	SCLC type	0		300
341	3814,5	70	stopgain	WT	WT	WT	WT	NSCLC pemetrexed type	0		0
372	3244	80	nonsynonymous SNV	WT	WT	WT	WT	SCLC type	0		260
380	1022,5	60	nonsynonymous SNV	WT	WT	WT	WT	NSCLC type	0		140
381	6718,5	50	nonsynonymous SNV	WT	WT	WT	WT	SCLC type	0		290
2002	919,5	90	WT	WT	WT	WT	WT	NSCLC type	0	0	300
61	2237	70	nonsynonymous SNV	WT	WT	nonsynonymous SNV	WT	SCLC type	0		230
144	2929	50	nonsynonymous SNV	WT	WT	stopgain	WT	NSCLC type	0		300
393	261	60	nonsynonymous SNV	WT	WT	nonsynonymous SNV	WT	NSCLC type	130		20
188	5882,5	70	WT	WT	WT	nonsynonymous SNV	WT	SCLC type	120		0
117	891	80	nonframeshift deletion	WT	stopgain	nonsynonymous SNV	WT	NSCLC type	110	80	30
176	1579,5	80	nonsynonymous SNV	WT	stopgain	nonsynonymous SNV	WT	NSCLC type	160		15
314	1253	70	WT	WT	nonsynonymous SNV	nonsynonymous SNV	WT	NSCLC pemetrexed type	200	160	0
229	2644,5	50	WT	WT	frameshift deletion	frameshift deletion	WT	NSCLC pemetrexed type	10		0
357	3143,5	50	frameshift deletion	WT	stopgain	WT	WT	SCLC type	150		0
367	1855,5	80	WT	WT	nonsynonymous SNV	WT	WT	SCLC type	5		200
8	3621,5	20	nonsynonymous SNV	WT	nonsynonymous SNV	WT	WT	Chemotherapy unknown	120		0
60	5604,5	90	nonsynonymous SNV	WT	nonsynonymous SNV	WT	WT	NSCLC type	80		300
130	6604,5	40	nonsynonymous SNV	WT	WT	WT	nonsynonymous SNV	NSCLC type	70		200
169	2326	70	nonsynonymous SNV	WT	WT	WT	WT	SCLC type	170		0

Table S10.2 (continued)

Case	Coverage	% tumor	Tp53	RB1	STK11	KEAP1	Men1	Chemotherapy	H-score RB1	H-score RB1 4H1	H-score P16
191	2368	70	nonsynonymous SNV	WT	WT	WT	WT	NSCLC type	120		0
221	1569	70	nonsynonymous SNV	WT	WT	WT	WT	NSCLC pemetrexed type	50		0
257	3060,5	80	nonsynonymous SNV	WT	WT	WT	WT	Chemotherapy unknown	135	65	0
261	3405,5	50	frameshift insertion	WT	WT	WT	WT	SCLC type	130		0
265	6870	60	stopgain	WT	WT	WT	WT	NSCLC type	200	200	0
316	3592,5	40	nonsynonymous SNV	WT	WT	WT	WT	Chemotherapy unknown	90		300
353	3297,5	80	nonsynonymous SNV	WT	WT	WT	WT	NSCLC pemetrexed type	170		0
2010	4221	40	nonsynonymous SNV	WT	WT	WT	WT	NSCLC type	160		200
80	1646	60	WT	WT	WT	WT	WT	NSCLC type	50	50	0
162	1455,5	90	WT	WT	WT	WT	WT	Chemotherapy unknown	200	120	200
275	2380	60	WT	WT	WT	WT	WT	NSCLC type	170	200	0
2004	1670	80	WT	WT	WT	WT	WT	SCLC type	140	60	0
6	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		300
59	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		300
67	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		300
77	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		300
108	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		0
139	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		300
170	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		300
197	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		300
198	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		300
199	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		300
200	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		UA.
220	2010 (duplicate low coverage)	70	Poor coverage	Poor coverage	Poor coverage	Poor coverage	Poor coverage	SCLC type	0		300
249	695,5 (duplicate low coverage)	70	Poor coverage	Poor coverage	Poor coverage	Poor coverage	Poor coverage	NSCLC type	0		300
254	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		200
280	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		300
288	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		300
311	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		300
312	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		300
329	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	Chemotherapy unknown	0		300
356	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC pemetrexed type	0		300
360	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	0		300
382	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	0		100

Table S10.2 (continued)

Case	Coverage	% tumor	Tp53	RB1	STK11	KEAP1	Men1	Chemotherapy	H-score RB1	H-score RB1 4H1	H-score P16
2005	5	80	Poor coverage	Poor coverage	Poor coverage	Poor coverage	Poor coverage	NSCLC pemetrexed type	0		300
159	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	10		0
53	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	150		0
51	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	Chemotherapy unknown	130		200
216	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	110		300
252	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	SCLC type	120		250
300	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	80		0
27	Insufficient DNA	Insufficient DNA	UA.	UA.	UA.	UA.	UA.	NSCLC type	130		0

Chapter 11

New insights into the molecular characteristics of rare neuroendocrine lung tumors: Carcinoid and large cell neuroendocrine carcinoma

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Abstract

Carcinoids and large cell neuroendocrine carcinomas (LCNEC) are rare subtypes of pulmonary neuroendocrine tumors. We reviewed recent next-generation sequencing studies to provide new insights into the biology of these tumors and integrate results with current knowledge.

Carcinoids generally contain a low mutational burden and few recurrent mutations, most frequently in chromatin remodeling genes (e.g. *MEN1),* and few affecting the *PI3K-AKT-mTOR* pathway. Aggressive disease has been related to chromothripsis/DNA-repair gene mutations as well as loss of OTP/CD44 with upregulation of *RET* gene expression. The low mutational burden in carcinoids suggests the presence of additional mechanisms leading to their development.

In LCNEC the mutational burden is one of the highest in cancer, possibly interesting for immune checkpoint blockade therapy. Similar to carcinoids, chromatin remodeling genes are frequently mutated. Different molecular subtypes are identified in LCNEC, in which bi-allelic inactivated*TP53* is found with either bi-allelic inactivated *RB1*, resembling hall marks of small cell lung cancer (SCLC), or *STK11/KEAP1* and/or *RAS* pathway mutations, resembling rather a non-small cell lung cancer (NSCLC) type. These subtypes likely respond differently to NSCLC chemotherapy. In combined LCNEC-NSCLC significant overlap in mutations are identified between components, providing further evidence that LCNEC may be clonally related with NSCLC.

Future studies should explore the relation between *mTOR* pathway mutations and response to *mTOR* inhibitor therapy in carcinoids. Targeting of activated oncogenes will unlikely advance treatment in the near future for LCNEC because the low frequency of activating mutations. However, chemotherapy treatment may be guided by identified molecular subtypes. Also, DLL3 and immunotherapy may provide alternative options for patient tailored therapy in LCNEC.

Introduction

Neuroendocrine lung tumors represent a subgroup of approximately 20% of lung cancer and can be subdivided into typical carcinoids (1.8%), atypical carcinoids (0.2%), large-cell neuroendocrine carcinoma (LCNEC) (3%) and small-cell lung carcinoma (SCLC) (15%)[1-3]. These subtypes can be separated by histopathological evaluation of mitosis and necrosis, and cell type for LCNEC *versus* SCLC[4]. Recently an increased incidence of carcinoids and LCNEC but a decreased incidence of SCLC has been reported[1-3,5]. Typical carcinoids generally occur around 45 (35-55) years of age and may even occur in children and adolescents, earlier than atypical carcinoid with average of 55 (45-65) years and LCNEC/SCLC with an average of 65 (55-75) years[1,6]. Patients with a typical carcinoid have a more favorable prognosis than patients with atypical carcinoids[1] and both subtypes have a better prognosis compared to patients with LCNEC or SCLC[7]. For the majority of carcinoid patients surgical management is possible whereas LCNEC and SCLC generally have metastatic disease at diagnosis, limiting the surgical options[1,6]. Nevertheless, when carcinoids have metastasized they are difficult to treat due to high resistance to radio- and chemotherapy[1]. Therefore, both carcinoids and LCNEC are in need of additional (systemic) therapies. To provide more insight, this review comprehensively evaluates recent next generation sequencing (NGS) studies highlighting common and different molecular characteristics, tumor progression, and (future) targets of treatment in carcinoid and LCNEC.

Genomic studies on rare lung neuroendocrine tumors

Carcinoids

Two studies extensively profiled carcinoids; Fernandez-Cuesta et al. investigated 69 carcinoids by RNA-seq and 44 tumor-normal tissue matched carcinoids by whole genome sequencing (WGS) or exome sequencing (WES) (Table 11.1A)[8]. Simbolo et al. used a discovery screen approach including tumor-normal tissue of 14 carcinoids by WES and 23 by high-coverage NGS (HCTS)[9]. Additionally, 51 formalin fixed paraffin embedded (FFPE) carcinoids were analyzed by a custom NGS panel. Armengol et al and Vollbrecht et al. also analyzed few cases using standard NGS cancer panels (Table 11.1A)[10,11]. Copy number variation (CNV) have been analyzed by SNP 6.0 (Fernandez-Cuesta et al. (n=54)) and by a NGS panel (Simbolo et al. (n=88)), Table 11.1B.

Table 11.1A Overview of studies that performed next generation sequencing in typical carcinoids, atypical carcinoids and LCNEC†

Study	Histology	Stage I-II disease	Tissue	Normal included	PA. rev	Tumor	Technique	Genes analyzed	Platform	Validation	Coverage	SNV Calling	AF
Fernandez-Cuesta	TC (59) AC (9) NOS CA (6)	83%	FF	Yes	Yes (2)	≥70%	WGS (29), WES (15), RNAseq (40),	All	HiSeq 2000	Sanger	WES T≈90 N≈30	Custom pipeline	Sign. from normal
Simbolo	TC (53), AC (35), LCNEC (27)	88%	FF (46) & FFPE (102)	Yes	Yes (5)	>70%	WES/HCTS: 23 TC, 14 AC, 5 LCNEC, 4 SCLC. TGT: 28 TC, 21 AC, 24 LCNEC, 29 SCLC.***	WES: all HCTS: 418, total exon coverage TGT: 88, hotspot & partly total exon coverage	HiSeq 2000 & Ion Proton	Multiplex PCR custom panel.	WES: T ≈100 N≈72 HCTS T≈ 850 N≈690	WES: GATK and MuTect. HCTS/TGT: Ion Reporter, custom pipeline	>20%
George	LCNEC (75)*	68%	FF	Yes	Yes (2)	>70%	WGS (11) WES (55), RNAseq (69)	All	HiSeq 2000	RNAseq, WGS and sanger.	WES T≈120 N≈90 WGS≈30 RNAseq≈30.	Custom pipeline	Sign. from normal
Armengol	TC (21) AC (4)	UA.	FFPE	?	Yes (1)	UA.	TGT: Ion AmpliSeq Colon and Lung Cancer Panel v1	22, hotspot, incomplete exon coverage	Ion PGM	In 4 T-N samples	UA.	Torrent Suite Software	UA.
Vollbrecht	TC (17) AC (17) LCNEC (19)	76% not stage IV	FFPE	No	Yes (2)	>50%	TGT: TruSeq Amplicon-Cancer Panel	48, (incomplete exon coverage)	MiSeq	No	T≈500-2000	Custom pipeline	>5%
Meder	LCNEC (19)	UA.	FFPE	No	UA.	UA.	TGT: Ion AmpliSeq custom panel	6, total exon coverage	UA.	No	UA.	UA.	UA.
Rekhtman	LCNEC (45)	84%	FFPE	Yes	Yes (3)	UA	TGT: MSK-IMPACT	241, total exon coverage	HiSeq 2500	No	T≈ 490 N: UA	Custom pipeline.	≥4%
Miyoshi	LCNEC (78)**	67%	FFPE	No	Yes (2)	>50%	TGT: Sureselect XT custom panel	244, total exon coverage	HiSeq 1500	Oncomine panel / Sanger,	T: ≈360	GATK (>50)	>10%
Karlsson	LCNEC (17)	88%	FFPE	No	Yes (?)	UA	TGT: TruSight Tumor gene panel	26, incomplete exon coverage	MiSeq	PCR for KRAS / Pyro for BRAF/EGFR.	UA.	MiSeq pipeline	>1%
Derks	LCNEC (79)	100% stage IV	FFPE	No	Yes (3)	>20%	TGT: GeneRead custom panel	5, total exon coverage	Ion Proton	No	T: ≈2850	Custom pipeline	>5%

† Karlsson et al. 2017 and Clinical Lung Cancer Genome Project (CLCGP) et al. 2013, data excluded as they are already included in George et al. (2017) and Karlsson et al. (2015); * 4 combined LCNEC-NSCLC, 12 combined LCNEC-SCLC, ** 10 combined LCNEC-NSCLC sampled both tumor components. *** For HCTS a customized AmpliSeq Comprehensive Cancer Panel (CCP) including 409 standard genes and 9 additional genes was used. For TGT AmpliSeq Cancer Hotspot panel v2 and three AmpliSeq custom panels including entire coding region of 45 genes were used

Abbreviations: TC, typical carcinoid, AC, atypical carcinoid; NOS CA, not otherwise specified carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; FF, fresh frozen; FFPE, formalin fixed paraffin embedded tissue; PA rev, pathology review; AF, allelic frequency; WES, whole exon sequencing; HCTS, high coverage targeted sequencing; TGT, targeted sequencing; SNV, Single nucleotide variant; WGS, whole genome sequencing; T, tumor tissue; N, normal tissue; RNAseq, RNA sequencing; UA, unavailable; GATK, Genome Analysis Toolkit; Sign, significant

Table 11.1B Overview of studies that analyzed copy number variation (CNV) in typical carcinoids, atypical carcinoids and LCNEC

Study	Histology	Technique	Bioinformatics	Validation
Fernandez-Cuesta	TC (45),AC (6), NOS CA (3)	SNP 6.0 (N=54)	Gauss-Newton approach (calibration) and circular binary segmentation method (segmentation)	Pyrosequencing
Simbolo	TC (53), AC (35), LCNEC (27), SCLC (33)	WES (N=20) & HCTS (N=46, 418 genes) & TGT (N=102, 13 genes*)	IonReporter 5.0, reference to 10 male normal DNA. Cut-offs not specified 3=low copy gain, ≥4 high copy gain	HCTS and TGT.
George	LCNEC (75)	SNP 6.0 (N=60)	Gauss-Newton approach (calibration) and circular binary segmentation method (segmentation) and CGRAS	RNAseq. data or sanger sequencing
Rekhtman	LCNEC (45)	MSK-IMPACT, HCTS (241 genes).	Custom pipeline.	No
Miyoshi	LCNEC (78)	HCTS (244 genes)	Calculated as ((gene Y sample coverage/total sample coverage)/average gene Y coverage all samples). ≥4 classified as gain, ≥10 as amplified	Additional SCLC cohort. Low sens. High spec. Only amplification/gains. Also oncomine panel/qPCR.
Karlsson	LCNEC (17)	Illumina 450K methylation beadchip	Log2 estimates from CpG probe intensity averaged to 12 normal tissues	No
Swarts	Carcinoid (10)	CGH arrays	Nexus Copy Number (6.0) software. Amplified cut-off of 0.6 on the log2-scale. Homozygous deletion as ≤-1.0 log2-scale	qRT-PCR

* Genes included: BCL2, FGFR1, MEN1, MYC, MYCL, PIK3CA, RB1, RICTOR, SDHA, SMAD4, SRC, TERT, and TP53

Abbreviations: TC, typical carcinoid, AC, atypical carcinoid; NOS CA, not otherwise specified carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; WES, whole exon sequencing; HCTS, high coverage targeted sequencing; TGT, targeted sequencing; SNP, single nucleotide polymorphism array; UA, unavailable; Sens, sensitivity; Spec, specificity; CGARS, cancer genome analysis by rank sums

Carcinoids are recognized by a low number of non-synonymous mutations (SNV) per million base pairs with an average of 0.4 mutations/Mb and are, compared to SCLC, slightly enriched for A:T>T:A trans-versions and A:T>C:G transitions but less G:C>A:T and C:G>A:T transversions. These differences emphasize a different (non-smoking) related development of carcinoid lesions[8]. Significant mutations reported by Fernandez-Cuesta et al. included *MEN1* (9%), *EIF1AX* (9%), and *ARID1A* (7%). The most frequently mutated genes identified by Simbolo et al. were *MEN1* (11%), *TP53* (10%), *KMT2C* (*MLL3*) (8%), *ARID1A* (6%), *DSCAML1* (5%), *KMT2D* (*MLL2*) (5%), *NOTCH2* (5%) and *PCLO* (5%). The majority of these genes are involved in chromatin remodeling; a process that controls gene expression in cells by opening/closing chromatin structure allowing/preventing transcriptional regulatory proteins to bind DNA. Fernandez-Cuesta et al. (40%) and Simbolo et al (46%) reported a high frequency of genes related to chromatin remodeling, all of the mutations identified by Fernandez-Cuesta et al. were mutually exclusive (P<0.0001).

The most frequently somatically mutated gene in carcinoid is *MEN1* (11-22%) usually accompanied by loss of heterozygosity (LOH)[8,9,12]. In 2% of pulmonary carcinoid patients a germline mutation in *MEN1* is identified[13]. *MEN1* is a histone modifier that acts as a scaffold protein assembling the *MEN1-MLL-PSIP1* ternary complex. Interestingly, another member of this complex, *PSIPS1*, has also been found recurrently mutated (5%) combined with *MEN1* in 13% (Fernandez-Cuesta et al.[8]). The *MEN1* complex inhibits the cell cycle by promoting the expression of *CDKN1B* ($P18^{Ink4c}$) and *CDKN2C* ($P27^{Kip1}$), both of them cell cycle inhibitor genes. *MEN1* has been found to be higher expressed in carcinoids than in normal tissue; however *MEN1* mutations are associated with reduced gene-expression and the mechanism is not through methylation of the CpG island in the promotor region[12].

Genes related to chromatin remodeling can be divided into histone covalent modifiers (methylation and acetylation) and ATP-dependent nucleosome remodeling genes that interact with the SWI/SNF complex. Histone methylation genes found mutated in carcinoid by Fernandez-Cuesta et al. included *SETDB1, KDM4, PHF8*, *JMJD1C*, *NSD1*, the polycomb complex, and by histone acetylation such as *BRWD3*/*HDAC5*[8,9]. In this context, the *KMT2 (MLL)* family of covalent histone modifiers was found mutated in 14% of carcinoids by Simbolo et al.[9]. Genes involved in the SWI/SNF complex were found mutated in 22% of carcinoids by Fernandez-Cuesta et al., most frequently including the *ARID1* gene family but also *BCL11A*, *SMARCA1/2/4*, *SMARCB1*, and *SMARCC2*[8]. Similarly, the *ARID1* gene family was mutated in 9% in the study of Simbolo et al.[9]. Finally, 8% of carcinoids were mutated in sister-chromatid cohesion genes, involved in DNA replication, including *DICER1*, *STAG1*, *ERCC6L*, and *NIPBL* while genes involved in E3 Ub ligases were found mutated in 18% (Fernandez-cuesta et al.)[8].

Tumor suppressor genes such as *TP53, RB1* and *ATM* are mutated in 6-10% of carcinoids (Fernandez-Cuesta & Simbolo et al.). Furthermore, Simbolo et al. identified a deletion or LOH in up to 13% for *RB1*, and 23% for *TP53* in carcinoids while mutations in *KRAS* have been identified in very few carcinoids[8-11]. The *PI3K/AKT/mTOR* pathway was found mutated in 2% of carcinoids by Simbolo et al.[9]; gain of function was described for *PIK3CA* (11%) and *RICTOR* (29%). No mutations were identified by Fernandez-Cuesta et al. in this pathway. By Sanger sequencing, recurrent kinase domain mutations in *PIK3CA* (exon 9 and 20) have been described in 13% of typical and 39% of atypical carcinoids[14]. These reported molecular alterations highlight an important role of the *PI3K/AKT/mTOR* pathway in a subgroup of carcinoids[9]. Although a couple of miRNA studies have been carried out in recent years[15-20], no recurrent and validated miRNAs have been identified in carcinoid subgroups or related to progression of disease as was also summarized in a table presented in a previous review[21].

Interestingly, a hyper-mutated profile has been observed in two carcinoids carrying POLQ gene mutations, making cells susceptible to DNA double-strand breaks and homologous recombination[9]. Furthermore, one carcinoid was identified with chromothripsis that affected genes related to chromatin remodeling[8]. Based on the results from Simbolo et al. it may be argued that atypical carcinoids (n=35) have a significantly higher frequency of mutations in *MEN1* compared to typical carcinoids (n=53) (6% *versus* 20%). Similarly, atypical carcinoids may have more frequently a gain of function of the genes *TERT, SDHA, RICTOR, MYCL* and *SRC*.

Large-Cell Neuroendocrine Carcinomas

Two studies have performed WES on LCNEC including 60 matched tumor-normal cases and 69 tumors evaluated by RNA-seq. (George et al.) and 3 WES analyzed cases (Simbolo et al., Table 11.1A)[9,22]. Additionally, four studies evaluated LCNEC using FFPE and (custom) NGS panels (Simbolo et al. (n=27), Rekhtman et al. (n=45), Miyoshi et al. (n=78), Derks et al. (n=79), Karlsson et al (n=17), Meder et al. (n=19) and Vollbrecht et al. (n=19))[9,11,22-27]. Only Simbolo et al. and Rekhtman et al. included matched tumor-normal tissue. For CNV analyses George et al. used SNP analysis while Miyoshi et al. applied a custom CNV panel. The other studies used varying bioinformatical approaches on data derived from NGS panels (Table 11.1B).

LCNEC harbors a mean of 64.7 mutations per sample (Simbolo et al.) and 8.5-10.5 SNV mutations per million base pairs (George et al. and Rekhtman et al.)[8,23] The mutation signature of LCNEC is strongly associated with smoking (C:G>A:T)[22]. The majority of genes frequently mutated in LCNEC are tumor suppressor genes with the mutation usually accompanied by LOH. George et al. reported significant mutations in *TP53* (92%), *RB1* (42%), *STK11* (30%), KEAP1 (22%), and *RAS*-pathway genes *KRAS/NRAS/HRAS* (7%).

Similar results were reported by Rekhtman et al. (78%, 31%, 33%, 29% and 29%), by Simbolo et al. (67%, 15%, 4%, 0% and 7%), Miyoshi et al. (71%, 26%, UA. (unavailable), UA., and 5%) and Derks et al. (85%, 47%, 10%, 17%, UA) but the frequency of mutations reported differed somewhat, respectively. George et al., Rekhtman et al. and Derks et al., reported that *RB1* mutations happen in a mutually exclusive way with mutations in *STK11* (all *P*<0.0001) and *KEAP1* (*P*<0.001 & not significant (2x)) and the *KRAS/NRAS/HRAS* pathway genes (including amplifications, *P*<0.049, *P*<0.0024 & UA.))[22,23]. Therefore, at least two subtypes of LCNEC have been suggested, separated into one with *TP53/RB1* mutations, also the hallmark of SCLC disease[28], and a second one with mutations in *STK11/KEAP1/RAF*-pathway genes more frequently found mutated in NSCLC[29]. *CDKN2A* ($P16^{ink4a}$) deletions were reported in 8% by George et al. and loss of this gene in 4% by Rekhtman et al.; mutually exclusiveness of *CDKN2A* deletion with *RB1* mutation were recently described in unpublished work[30].

Oncogenic amplifications identified by George et al. included *MYC* family members (*MYC* (3%), *MYCL1* (10%)) as well as for *NKX2-1* (*TTF1*, 10%), *FGFR1* (3%) and *IRS2* (4%). Rekhtman et al. identified similar amplifications in *MYC* family members (*MYC* (13%), *MYCL1* (7%) and MYCN (2%)) as well as for *NKX2-1* (20%), *FGFR1* (4%) and *IRS2* (4%) while unique amplifications included amongst others *SOX2* (11%) and *CCNE1* (9%)[23]. Simbolo et al. also identified high copy gains for *MYC* (14%) and *FGFR*1 (4%) and unique gains in *RICTOR* (11%) while Miyohsi et al. identified several copy gains but merely amplification for *KRA*S (3%).

Other molecular alterations frequently identified in LCNEC include mutations of *NOTCH* family members *NOTCH1* (10%, 16%, 0%, and 9%)) NOTCH2 (3%, 2%, 15%, and 1%), NOTCH3 (7%, 8%, NR, and 4%) and *NOTCH4* (3%, 16%, 0% and NR). Similar as in carcinoid, genes related to chromatin remodeling are recurrently mutated including *MEN1/PSIP1* (7%, 4%, 4% and 0%), *ARID1A/B* (12%, 15%, 4% and 5%)), MLL1 (0%, 8%, 4% and 1%), *MLL2* (0%, 13%, 19% and 9%) and *MLL3* (0%, 13%, 7% and 12%). Simbolo et al. suggested that carcinoids and high-grade neuroendocrine carcinomas (SCLC and LCNEC) have similar mutation frequencies in these remodeling pathways (46% *versus* 55%, *P*=0.32) an even higher frequency of mutations in LCNEC was reported by Rekhtman et al. (78%)). More recently, also a carcinoid like subtype of LCNEC has been suggested having lower Ki-67 expression along with a *MEN1* mutation, but the existence of such a subtype requires further evaluation[23].

PI3K-AKT-mTOR mutations have been identified in 49% of LCNEC by Rekhtman et al. while Miyoshi et al. reported a frequency of 15%. Except for recurrent *PTEN (*7% George et al.) and *PI3KCA* mutations (11%, Simbolo et al.), frequent mutations in this pathway have yet to be confirmed by the other studies. Finally, several mutations unique to LCNEC were reported by George et al. including *ADAMTS* 12 (20%), *ADAMTS2* (15%),

GAS7 (12%) and *NTM* (10%) the latter two are related to neuroendocrine differentiation and neurogenesis. Simbolo et al. reported that *SMARCA2* (11%) gene mutation was unique for LCNEC. Rekhtman et al. reported unique mutations for *NTRK2/3* (19%) previously also reported in LCNEC[31] while Miyoshi et al. reported that *LAMA1* (10%), PCLO (6%) and MEGF8 (5%) were significantly more frequently mutated in LCNEC compared to SCLC.

Gene-expression analysis of n=8 and n=28 LCNEC tumors has shown that LCNEC can be subdivided into different subtypes, only partly clustering with SCLC[32,33]. Fernandez-Cuesta reported that in LCNEC (n=66) with molecular SCLC-type mutations (i.e. *TP53/RB1*), high *NOTCH* gene expression was observed, with the correspondent low expression of the Achaete-Scute Homolog 1 (*ASCL*-1) gene and the neuroendocrine genes Chromogranin-A and Synaptophysin. In LCNEC with NSCLC like mutations, high *ASCL*-1 gene expression was observed along with higher expression of neuroendocrine genes and low *NOTCH* gene expression. Karlsson et al. (n=14) also reported different LCNEC subtypes by gene expression analyses. Contrary to George et al. they reported high neuroendocrine gene expression in LCNEC with RB1 mutations and SCLC like gene expression patterns (n=11), whilst NSCLC like LCNEC (n=3) showed modest neuroendocrine gene expression[34].

An overview of the most common and unique molecular alterations identified in carcinoid and LCNEC, along with those from SCLC previously reviewed by Swanton et al.[29], are presented in Figure 11.1. Additionally, most frequently identified in carcinoid and LCNEC reported in at least two studies (NGS and/or targeted analyses) is presented in Table 11.2. In carcinoid the low frequency of mutated tumor-suppressor genes and relatively low mutational burden but high frequency of mutations in genes related to epigenetic mechanisms indicates different mechanisms resulting in increased proliferation and decreased apoptosis than LCNEC and SCLC. In contrast, LCNEC is recognized by a very high mutational load and particularly tumor suppressor gene mutations, i.e. *TP53* mutations in combination with either *RB1* mutations or *STK11/KEAP1*/RAS-pathway mutations. The relevance of these molecular subtypes requires further evaluation in the near future. Since epigenetic mutations occur in about half of all neuroendocrine tumor subtypes and the mutational burden increases with advancing histopathological stage, it could be speculated that tumor subtypes with histological features close to the diagnostic cut-off points might show transition to a higher grade.

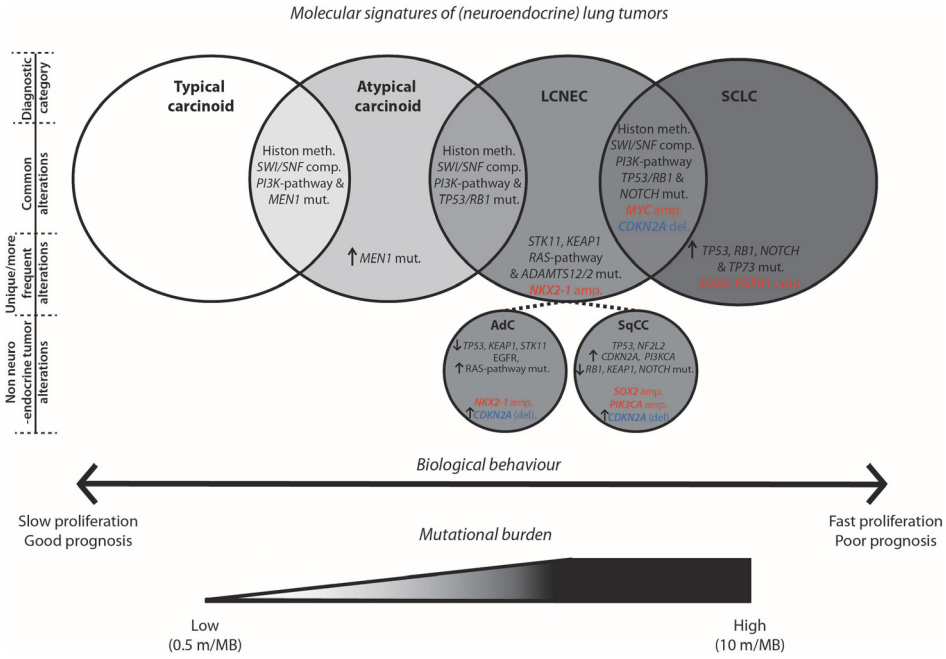

Figure 11.1 An overview of common genomic alterations overlapping and unique to typical carcinoid, atypical carcinoid, LCNEC and SCLC is given. Additionally, alterations for the NSCLC subtypes AdC and SqCC are shown
Abbreviations: MB, mega base; meth, methylation; comp, complex; mut, mutation; amp, amplification; del, deletion, AdC, adenocarcinoma, SqCC, squamous cell carcinoma

Cells of origin in rare neuroendocrine lung tumors

The majority of pulmonary neuroendocrine tumors likely develop from normal neuroendocrine cells (Kulchitsky cells) present in the large bronchi and smaller bronchiole where they tend to form neuroendocrine bodies (NEBs)[35,36]. Some non-neuroendocrine cancers may also develop neuroendocrine features such as NSCLC with neuroendocrine differentiation (NSCLC-NED). The location of neuroendocrine tumors in the lung appears not to be random, i.e. a proximal-to-distal distribution is observed moving from the trachea to the alveolar compartment. SCLC and typical carcinoid generally occur central (in the large bronchi), atypical carcinoid central and peripheral (bronchioles/alveolar duct/alveoli) and LCNEC generally peripheral[37]. These differences in anatomic location may be caused by diverse cell(s) of origin.

Table 11.2 Molecular alterations identified in published studies evaluating carcinoids and LCNEC using next generation sequencing†

Type of molecular alteration	Carcinoid	LCNEC
Cell Cycle mutations	*TP53 (5%), RB1* (2%), *APC* (4%)	*TP53* (81%), *RB1* (33%), *ATM* (4%)
RAS/MAPK pathway	*KRAS* (3%), *EGFR** (2%**)	*STK11* (18%), *KRAS/NRAS/HRAS* (11%), *ERBB4* (6%), *BRAF**** (2%), *EGFR**** (2%)
PI3K-AKT-mTOR pathway:	*PIK3CA* (2%)	*PTEN* (5%), *PIK3CA* (3%), *NF1* (5%), *INSR* (3%), *TSC2* (2%), *RICTOR* (2%)
Oxidative stress response:	-	*KEAP1* (21%)
Cell migration/adhesion:	-	*ADAMTS12** (20%), *ADAMTS2** (15%), *EPHA5** (7%), *FAT1* (6%),
Neurogenesis/endocrine differentiation:	-	*GAS7** (12%), *NTM** (10%), *PCLO* (5%), *PTPRT** 10%, *CSMD3** (7%) *NOTCH* family: *NOTCH1* (10%), *NOTCH2* (4%), *NOTCH3* (4%), *NOTCH4* (8%)
Epigenetic regulation:		
Chromatin remodeling	*MEN1* (11%), *EIF1AX** (9%), *PSIP1** (5%), *MLL2** (3%), *MLL3** (5%), *ARID1A* (6%), *ARID2* (2%),	*MEN1/PSIP (3%), ARID1A*/B (9%), *MLL1* (3%), *MLL2* (9%), *MLL3* (8%), *ATRX* (4%)
SWI/SNF complex	*SMARCA4* (3%), *SMARCB1* (3%),	*SMARCA4* (5%),
Histone (methyl/acetyl) transf.	*SETD1B** (5%),	*CREBBP/EP300* (7%)
E3 ubiquitin-protein ligase	*HERC2** (5%), *SEC31A** (5%), *WDR26**(5%)	-
Amplifications	-	*NKX2*-1 (14%), *MYC* family: *MYC* (7%), *MYCL1* (9%) & *MYCN* (1%) *FGFR1* (3%), *SOX2* (3%), *IRS2* (3%)
Deletions	*RB1** (6%), *TP53** (3%)	*RB1* (9%), *CDKN2A* (6%)
Commonly altered pathways	Chromatin remodeling	Cell Cycle control, Oxidative stress response, Neuroendocrine differentiation, *PI3K* and *RAS* signaling, Chromatin remodeling
Advised routine screening	-	*BRAF* (2%)***, *EGFR* (2%)***

† Averaged results of identified gene mutations/amplifications of studies investigating carcinoids and LCNEC using next-generation sequencing. Complete overview of identified gene alterations for each study can be found in the supplementary data file 1. * Gene alteration reported in a single study, not confirmed or investigated by other studies. ** No classical activating *EGFR* exon 19 deletion or exon 21 mutations identified. *** Including few activating *BRAF* V600 and activating *EGFR* exon 19 deletions / 21 substitutions

Abbreviations: TC, typical carcinoid, AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; transf, transferases

Carcinoids likely develop from neuroendocrine cell hyperplasia and carcinoid tumor lets, identified as pre-cursor lesions, and can be associated with a cluster of symptoms and pathological findings referred to as diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH)[36,38]. Pulmonary neuroendocrine cell hyperplasia can arise within a spectrum of diseases, including carcinoid, adenocarcinoma or as a reaction to inflammation[38,39]. Hence, studies evaluating the development of carcinoid from these possible different causes of neuroendocrine cell hyperplasia would be necessary to better understand tumorigenesis. Interestingly, the *Ortopedia Homeobox* gene was recently identified as a specific marker for pulmonary carcinoid and appears to be highly expressed in pulmonary neuroendocrine cell hyperplasia but not in neuroendocrine cells and NEBs[40,41].

Genetically engineered mouse models for neuroendocrine carcinomas were recently reviewed and included, amongst others, knockouts for *RB1*/TP53 alone, or combined with *PTEN* or *p130* in basal, neuroendocrine, clara and AT-II cells[42]. When neuroendocrine cells were targeted, mostly SCLC developed in these mice, except for the *RB1*/*TP53*/*PTEN* knockout in which also LCNEC developed. Interestingly, in the *RB/TP53/p130* knockout that targeted all lung cells, LCNEC much more frequently developed. These experiments may suggest that LCNEC can develop from neuroendocrine cells but even more frequently from non-neuroendocrine cells.

Previous studies have hypothesized that LCNEC is clonally related to NSCLC and SCLC[22,23]. In combined NSCLC-LCNEC tumor samples (n=10 and n=12), up to 99% of identified mutations were observed in both the neuroendocrine and non-neuroendocrine components[24,43]. The high mutational correspondence between combined LCNEC components suggests a monoclonal developmental origin, also recently described for other (non-pulmonary) combined neuroendocrine carcinomas[44,45]. Nevertheless, if combined-LCNEC has a common origin, the next question would be what the primary component would be. Thus far, only trans-differentiation (or dedifferentiation) of a non-neuroendocrine NSCLC towards high-grade neuroendocrine carcinoma has been described. This trans-differentiation can occur in NSCLC with activating *EGFR* mutations (exon 19 deletion or exon 21 L858R) as a resistance mechanism to *EGFR* tyrosine kinase inhibitor treatment (3-14%)[46-49]. The main mechanism for the trans-differentiation has been ascribed to (bi-allelic) loss of *RB1* function, previously suggested to also modulate neuroendocrine gene expression[50], not present in the original NSCLC but occurring in the resistant SCLC/LCNEC tumor[51,52]. In LCNEC two combined adenocarcinoma with LCNEC have been described, in one case the identified *RB1* mutation was enriched in the LCNEC component but not the adenocarcinoma component[24,53]. Finally, elevated NOTCH can reverse the neuroendocrine phenotype observed in mice models and by human derived tumor gene expression analysis in SCLC[22,28]. Loss of *NOTCH* therefore has been

suggested as an alternative pathway of non-neuroendocrine cells to develop SCLC[26], also observed in LCNEC with NSCLC subtype (i.e. STK11 or KEAP1 mutation)[22].

Altered genes that inform on progression of disease

In terms of prognosis, some studies evaluated patients having a carcinoid a good *versus* a poor prognosis (i.e. rapid death of disease, n=10 discovery, n=54 validation)[40,54]. In carcinoids with a poor prognosis the proto-oncogene *RET* was upregulated along with genes involved in the cell cycle control such as *ASPM*, *BIRC5*, *BUB1*, *CASC5*, *CEP55*, *FANCA*, *HIST1H3B*, *ORC6L*, *RCC1*, and *UBE2C*[40,55]. Important downregulated genes included *FOLR1*, *FOLR3*, *DLG2*, *B3GAT1*, *KIRREL3*, and *FXYD2* all localized at chromosome 11q, previously found as having prognostic value when lost[54]. Only two genes, *OTP* and stem cell marker *CD44,* could be validated on an additional series of n=288 carcinoids for protein expression and their loss of expression was confirmed to be of a worse prognostic value, also by others[40,41,56]. Other studies Indicated *MEN1* mutations, a high number of chromosomal alterations, and larger chromosomal alterations with poor prognosis in carcinoids[9,12,54,57]. Mechanisms causing chromosomal alterations and/or tumor instability might be related to histopathological grade progression but thus far this has not been proven.
Advanced disease LCNEC (n=13) maybe characterized by a higher frequency of *MYC* family genes copy number gain (*MYC* (15%), *MYCL1* (23%) and *MYCN,* (*8%)* versus early stage LCNEC (n=65) *(*0%/8%/0%, *P*=0.002), suggesting that this event could be related to progression of disease[24]. Also, amplification of *NFIB* may be related to progression of disease in LCNEC as suggested by a recent mice model study in SCLC and investigation of *NFIB* in LCNEC (n=6)[58]. Furthermore, a recent NGS study in metastatic LCNEC identified a higher frequency of *RB1* mutations compared to previous studies on surgically resected LCNEC (±45% *versus* ±30%)[27]; *RB1* may be related to progression of disease in LCNEC.

An illustration of the cells of origin and genes related to progression of disease in carcinoid and LCNEC is provided in Figure 11.2. Carcinoid tumors likely arise from neuroendocrine cells and related neuroendocrine cell hyperplasia, and the genes *OTP/CD44* may play an important role in carcinoid development. The unique clinical presentation of LCNEC (peripheral tumor location similar to NSCLC) and the more frequent development of LCNEC when not targeting neuroendocrine cells in genetically engineered mice models may indicate, in a subset of tumors, non-neuroendocrine cells of origin (i.e. bronchoalveolar stem cell / ATII cell). This is also supported by identification of LCNEC with a more NSCLC molecular subtype and the high correlation

between mutations identified in combined LCNEC-NSCLC components. Finally, it can be hypothesized that LCNEC may develop neuroendocrine features by *RB1* mutations or upregulation of neuroendocrine transcription factors such as ASCL-1 by downregulation of NOTCH[26].

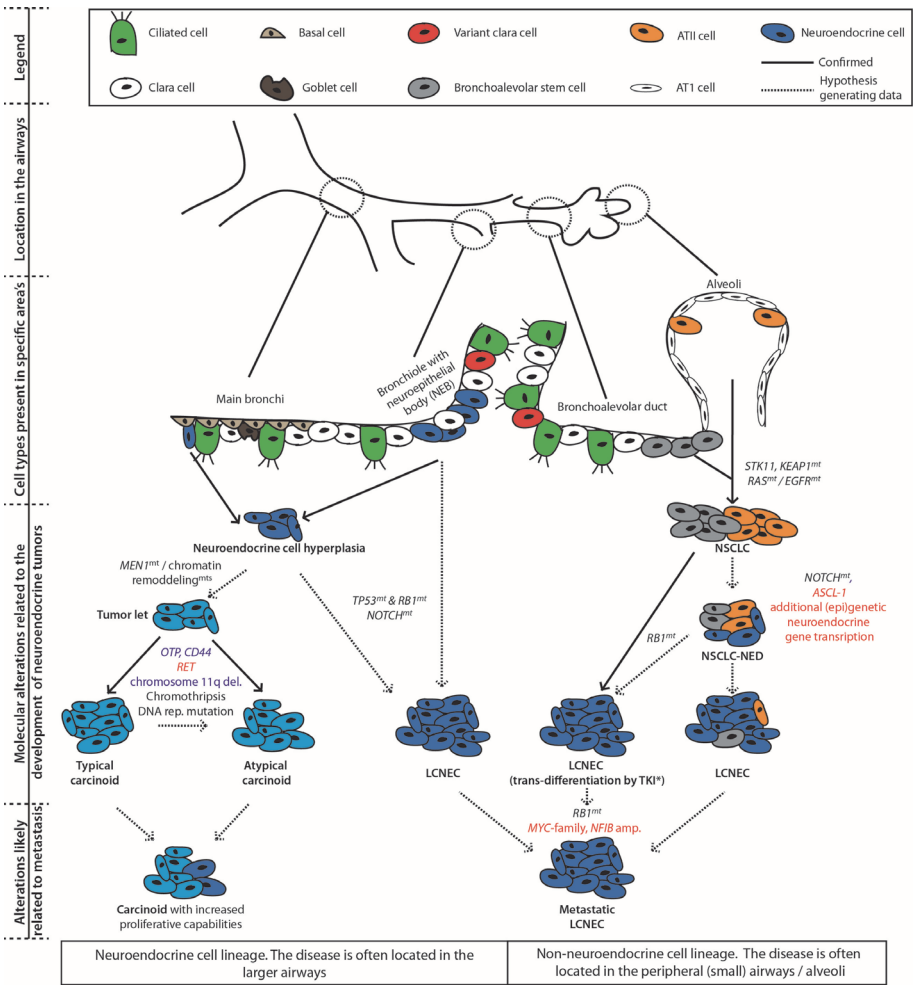

Figure 11.2 A putative overview of molecular alterations in proposed pulmonary cells of origin leading to carcinoid and LCNEC, molecular alterations related to metastatic disease are given
Abbreviations: alt, alternative ; morph, morphology ; TKI, tyrosine kinase inhibitor ; del, deletion; amp, amplification; mt, mutation; NSCLC-NED, non-small cell lung carcinoma with neuroendocrine differentiation; LCNEC, large cell neuroendocrine carcinoma; rep, replication

New treatment possibilities based on genomic data

Carcinoid

In terms of therapeutic targets, a few targetable mutations have been identified in carcinoids in amongst others the *PI3K/AKT/mTOR* pathway. *PI3K* pathway inhibitors recently have shown clinical effectivity in lung carcinoids with a prolonged progression free survival of 11.0 (9.2-13.3) months compared to 3.9 (3.6-7.4) months *versus* supportive care[59]. Nevertheless, studies comparing biomarkers for expression of this pathway to predict response to *mTOR* inhibitors have yet to be provided. Several additional inhibitors are currently being investigated in carcinoids, including Cabozantinib (*c-MET*, *VEGFR2*), Regorafenib (*VEGFR2-TIE2*, multi receptor tyrosine kinase inhibitor), Nintedanb (*VEGFR*, *FGFR*, *PDGFR*), Sufatinib (*VEGFR*, *FGFR1*), Carfilzomib (20S proteasome inhibitor) and Ibrutinib (Brutons tyrosine kinase inhibitor) and were recently extensively reviewed[60]. Thus far two case reports have reported an *EML4-ALK* rearrangement in patients with atypical carcinoid only one patient responded to Crizotinib (*ALK*, 1st generation) treatment[61,62]. Summarizing we can conclude that based on the currently available genomic studies, no other targetable mutations than that of *PI3K-AKT-mTOR* pathway genes have been reported, making effective treatment of carcinoid with small molecule inhibitors rather unlikely.

LCNEC

In the context of treatment, reportedly two third of LCNEC show molecular alterations that might be targets for future drug treatment (n=45, Rekhtman et al.)[23]. They occur more often in *RB1* wild-type LCNEC (84%) than in *RB1* mutated LCNEC (50%)[23]. Currently, targetable mutations include those in *EGFR* (±2%), *BRAF* (±1%) and amplification of *FGFR1 (±3%)*. Recent unpublished work suggested response to a BRAF inhibitor in a V600E (G469R) *BRAF* mutated LCNEC[63]. Furthermore, three case reports have described clinical activity for Gefitinib or Icotinib (*EGFR*, first generation) in LCNEC with activating *EGFR* exon-19 deletions[64,65]. Treatment with Crizotinib for a LCNEC with *EML4-ALK* rearrangement was ineffective[66]. Hence, standard routine screening for activating (targetable) mutations should be encouraged.

Genes related to the *PI3K-AKT-mTOR* pathway are occasionally mutated in LCNEC (Table 11.2). The results from a phase II clinical trial combining Carboplatin-Paclitaxel chemotherapy with daily Everolimus maintenance treatment (mTOR, first generation) showed encouraging results[67]. However, survival results were only slightly better than those reported for an European chemotherapy phase II trial[68] and a retrospective study

with taxane chemotherapy[69]. Evaluation of *RB1* status and *mTOR* pathway genes might be interesting in order to select LCNEC patients with improved treatment response.
A humanized monoclonal antibody directed against *DLL3,* a ligand of *NOTCH*, conjugated to a DNA-damaging pyrrolobenzodiazepine dimer toxin has recently been tested as an anti-tumorigenic drug for LCNEC and SCLC. *DLL3* expression was observed in 65% of LCNEC using immunohistochemistry. In a recent phase 1A/1B 2nd/3rd line dose escalation and expansion study for *DLL3*-antibody therapy in SCLC and LCNEC, evidence for clinical activity was provided, particularly for high *DLL3* expressing tumors [70,71]. Hence, DLL3 targeted antibody drug therapy may be of interest for future treatment of LCNEC.
The observation that LCNEC frequently shares mutations with SCLC (*TP53/RB1)* and NSCLC (*STK11/KEAP1/RAS*-pathway genes) may help to unravel observed heterogeneous response to chemotherapy in LCNEC[23]. Recent analysis of 79 LCNEC identified that LCNEC with NSCLC type mutations have superior outcome when treated with NSCLC type chemotherapy (platinum-gemcitabine or paclitaxel chemotherapy). No differences in chemotherapy treatment outcome were observed for LCNEC with SCLC (*RB1*) mutations[27]. Interestingly, in a prospective SCLC study *RB1* wild-type status was correlated with resistance to SCLC type (platinum-etoposide) chemotherapy[72].

Immunotherapy

Several studies are currently evaluating immune checkpoint blockade therapy targeting programmed cell death ligand (PD-L1) receptor with anti-PD(L)1 inhibitors, including combined CTLA-4 (immune checkpoint blocker, Tremelimumab) and PDL-L1 (Durvalumab) antibody treatment (phase II, EudraCT: 2016-002858-20) and PD-1 antibody treatment (PDR001, phase II, NCT02955069). In typical (n=95) and atypical carcinoids (n=11) the expression for PD-L1 using the E1L3N antibody is absent (0%, H-score >1), but only very few metastatic carcinoid cases have been analyzed[73,74]. Recently, it was reported that the mutational tumor burden is related to response to immunotherapy[75,76]. Considering the very low mutational burden in (typical) carcinoids, these likely will not respond to PD-L1 therapy. However, patients with carcinoids showing chromothripsis or a hypermutation profile may be more prone to respond requiring further evaluation.
In LCNEC, a retrospective multicenter cohort reported ten patients (50% 3rd line treatment, all in good performance (0-1)). Checkpoint blockade therapy showed encouraging results with six partial responses and long progression free survival (median 57 weeks, [24-57]) in this highly selected patient cohort[77]. Currently a PD-1 inhibitor (Pembroluzimab) is being investigated in LCNEC (phase II, NCT02939651). PD-L1 in early stage LCNEC may show expression in up to 34% of tumors (n=72)[78]. However, expression was only 10.4% and 0% in other cohorts (n=106 and 11)[73,74]. The mutational burden is

extremely high in LCNEC (±10/Mb) and was related to PD-L1 expression in LCNEC, therefore some LCNEC's may show favorable response to immunotherapy[22,23,78].

Conclusions

Newly attained insights into the molecular background of neuroendocrine tumors still support the separation of typical and atypical carcinoids from LCNEC and SCLC[37]. Carcinoids are recognized by frequent mutations in chromatin remodeling genes but molecular alterations are infrequent. Despite extensive genomic analysis targeted treatment in patients with these tumors therefore remains limited. Consequently, future studies should further unravel other mechanisms possibly leading to cancer development such as evaluation of the methylation pattern of carcinoids. Molecular available data show that LCNEC should be considered as a unique group of neuroendocrine tumors with high mutational burden. Separation of LCNEC into a SCLC type (co-inactivated *TP53* and *RB1*) and NSCLC type (*STK11/KEAP1/KRAS* with *RB1* wild-type) may be clinically relevant for the choice of chemotherapy regimen. Possible targetable mutations have been identified in carcinoids and LCNEC including the *PI3K-AKT-mTOR* pathway and specifically for LCNEC mutations in *EGFR* and *BRAF* in a small subpopulation requiring routine screening in clinical practice. Nevertheless, the efficacy of targeted treatment can be debated in case *RB1* is inactivated as the key restriction point of activation of the cell cycle. *DLL3* antibody therapy may be relevant for LCNEC and requires further investigation. Finally, immunotherapy might be an option for treatment of LCNEC and possibly carcinoids with a hyper-mutation profile, although clinical response for checkpoint blockade therapy in these rare tumors currently is still lacking.

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Chapter 12

General discussion

Large cell neuroendocrine carcinoma (LCNEC) is a rare subtype of lung cancer with an incidence of 1-3%. Its rarity together with the fact that the disease is difficult to diagnose on a biopsy specimen makes it a complex type of lung cancer[1-4]. Consequently, clinical trials evaluating chemotherapy treatment in metastatic LCNEC have failed to include an appropriate amount of patients[5,6]. As a result, evidence based treatment guidelines don't exist. Therefore, we aimed to comprehensively evaluate the diagnosis and treatment of LCNEC (chapters 2-6). Furthermore, our aim was to optimize the diagnosis of LCNEC on a biopsy specimen (chapter 7-8). Also, a retrospective analysis was performed on chemotherapy treatment in patients with metastatic LCNEC and tumor specimens of LCNEC were carefully revised for precise diagnostic classification (chapter 9). Finally, recently identified genomic LCNEC subtypes were examined in tumor specimens and subsequently correlated with chemotherapy treatment outcome results (chapter 10-11). The results presented in this thesis may lead to an improvement of the clinical management of patients with LCNEC, and provides diagnostic recommendations necessary to allow an effective multi-center randomized clinical trial evaluating optimal management of metastatic LCNEC.

1. Diagnosis of LCNEC in the daily pathology practice

1.1 Frequency of LCNEC diagnoses on biopsy specimens

In lung cancer, the first diagnostic step after imaging is bronchoscopy where relatively small biopsy samples are obtained. Assessment of defining diagnostic features such as specific morphology (e.g. adeno, squamous or neuroendocrine) may be challenging on these small biopsies[7]. According to the most recent World Health Organization classification (WHO 2015), LCNEC can be diagnosed on biopsy specimen when both neuroendocrine morphology and neuroendocrine differentiation, identified by immunohistochemistry (IHC), are observed along with high-grade non-small cell cytomorphological features [8]. The WHO diagnosis can then be concluded and is defined as 'non-small cell lung cancer (NSCLC), possible LCNEC'. The WHO classification of 1999 and 2004 (used in practice until 2015) did not support a diagnosis of LCNEC on a biopsy specimen[9]. Nevertheless, pathologists have established LCNEC diagnoses in ≥50% on both biopsy and cytology specimens since 2003 as shown in chapter 5. A recent study indicated that in the current WHO classification LCNEC is actually an umbrella term comprising cases overlapping with SCLC, NSCLC and carcinoids with >10 mitosis/ 2mm2[10]. The use of immunohistochemistry may support in the distinction of LCNEC from SCLC and carcinoids.

1.2 Overlapping diagnoses: LCNEC *versus* NSCLC

In **chapter 8**, we compared the diagnosis of pre-operatively taken biopsy specimen to that of the matched surgical resection specimen in LCNEC. The results provided an argument that the difficulty a pathologist may perceive in diagnosing LCNEC in small specimens is largely due to the small cumulative sample size, prohibiting the presence and/or recognition of neuroendocrine morphology in 50% op biopsy specimen; making the identification of LCNEC on biopsy specimen almost impossible which often lead to a NSCLC diagnosis. This is an important problem requiring attention of both pathologists and physicians in clinical practice, especially since this diagnostic overlap can impact clinical treatment and patient outcome. When non-squamous NSCLC is diagnosed, the currently advised chemotherapy is platinum-based-pemetrexed treatment[5]. In **chapter 9**, evidence for an inferior treatment outcome in patients with metastatic LCNEC treated with platinum-pemetrexed chemotherapy *versus* platinum-gemcitabine chemotherapy is provided, albeit retrospectively. Not only was it shown that platinum-pemetrexed chemotherapy responds poorly in the few studies evaluating LCNEC treatment[11], genes related to resistance to pemetrexed chemotherapy also have been shown to be upregulated in LCNEC[12-14].

To improve the recognition of LCNEC, we suggest implementing staining of ≥2 out of three neuroendocrine IHC markers (chromogranin-a, synaptophysin and/or CD56 (NCAM)) as a surrogate marker for neuroendocrine morphology in the context of an undifferentiated NSCLC on a biopsy specimen (**chapter 8**). Currently, the WHO advises not to stain for these markers when neuroendocrine morphology is not observed[15,16] (**chapter 7**) because neuroendocrine IHC staining is sometimes also positive in NSCLC (8-33%) without any prognostic relevance[17-23]. Nonetheless, double staining of neuroendocrine markers is reported in less than 1% and triple in none NSCLC[22]. Double staining is observed in ≥80% of LCNEC, and triple in ≥60%[3,24]. In the near future, the reported results require validation in a cohort of LCNEC, adenocarcinomas and squamous cell carcinomas with (focal) neuroendocrine staining, to determine the true diagnostic value of these additional staining's on biopsy specimens.

1.3 Overlapping diagnoses: LCNEC *versus* SCLC

To distinguish LCNEC from SCLC solely by cytomorphological criteria has, and probably will remain, to be an important diagnostic problem for pathologists. This is illustrated by the significant inter-observer variation in high-grade neuroendocrine carcinoma[10,25-28], and by the cases originally diagnosed as NSCLC/LCNEC revised as SCLC in **chapter 8**. The diagnosing obstacle comes from several factors such as tissue crush artefacts, reported

distorted cytological features of SCLC on larger tissue samples[29], and overlap in cell and nuclear size between LCNEC and SCLC demonstrated by morphometric studies[30,31]. Nevertheless, in **chapter 8**, only a small proportion of biopsy specimen diagnosed as LCNEC were eventually diagnosed as SCLC in the resection specimen.
Previously, several potential diagnostic markers distinguishing LCNEC from SCLC (*BRAI-3*, *VIL-1* and *CDX-2*) have been reported but these have not been related to clinical outcome and a subgroup of neuroendocrine carcinomas remained indistinguishable[32-34]. A recent study summarized possible relevant immunohistochemical markers useful to discriminate LCNEC from SCLC including RB1 and P16[10]. Several studies have identified a difference in the regulation of the *RB1* and *CDKN2A* ($P16^{ink4a}$) genes between LCNEC and SCLC[24,34-38]. In these studies, RB1 shows positive IHC staining in LCNEC in 33-55% whilst this is ≈10% for SCLC. In **chapter 10** we suggest that RB1 and P16 IHC analysis may be valuable to identify subtypes of high-grade neuroendocrine carcinomas, since having retained expression for RB1 and/or loss of P16 can lead to a favorable response to NSCLC type chemotherapy. In addition, application of RB1 IHC staining may be useful for pathologists to identify at least a subgroup of LCNEC.

1.4 Overlapping diagnoses: LCNEC *versus* carcinoid

Previous studies have described diagnostic overlap between high-grade neuroendocrine carcinomas and carcinoid, especially in crushed biopsy samples[39]. In our evaluations, we only found a limited overlap. Nevertheless, mitosis and necrosis were commonly difficult to evaluate in LCNEC biopsy specimen (**chapter 8**) and were often not or only partly (without a mitotic index) described in LCNEC pathology reports (**chapter 6**). The diagnosis of an (atypical) carcinoid as LCNEC may impact clinical outcome as shown in **chapter 4** and highlighted by the improved prognosis of LCNEC cases revised as carcinoid in **chapter 10**. To increase the diagnostic discrimination of LCNEC from carcinoid in biopsy specimen, the usage of proliferation markers such as Ki-67 has been suggested[39]. Thus far, Ki-67 evaluation by so-called eyeball method (i.e. estimation of the pathologist by light microscopy) seems to be the most pragmatic approach in limited tissue in daily practice with a cut-off at ≥20% to define high-grade neuroendocrine carcinoma[40,41]. Furthermore, application of synoptic reporting protocols, such as that recently implemented by PALGA[42], as a support tool to evaluate and describe all WHO criteria, may help to decrease the (few) numbers of LCNEC diagnosed as atypical carcinoid in daily practice but revised by a panel of pathologists (**chapter 6**).

1.5 Practice of non-WHO nomenclature in high-grade neuroendocrine carcinomas

The difficulties experienced by pathologists to categorize cases at the borderline between distinct classes, may lead to the use of non-WHO-terminology as a best approximation. Ambiguous (non-WHO) nomenclature is often (20%) used in the daily pathology practice to describe amongst others neuroendocrine carcinomas of uncertain type on biopsy/cytology specimen (**chapter 5**). The application of immunohistochemical markers such as RB1 and P16 may overcome this and enables a clearer discrimination of high-grade neuroendocrine carcinoma types (i.e. SCLC *versus* LCNEC), relevant for chemotherapy treatment. Furthermore, nomenclature such as '*NSCLC with neuroendocrine features'* or *'differentiation'* is widely used for LCNEC (**chapter 5** and **chapter 9**). However, such nomenclature is unclear for physicians (**chapter 5**) and not advised by the current WHO (2015, **chapter 7**). Usage of such nomenclature likely leads to differences in chemotherapy treatment; '*neuroendocrine carcinoma'* was generally treated with SCLC-type chemotherapy whilst '*NSCLC with neuroendocrine features'* or '*differentiation'* was often treated with NSCLC type chemotherapy (**chapter 9**). More importantly, NSCLC type chemotherapy often included platinum-pemetrexed of which it was shown to have a negative effect on outcome in patients with LCNEC (**chapter 9**).
Physicians treating patients with LCNEC are thought to have insufficient awareness of the difficulties pathologists encounter when diagnosing these tumors. Similarly, pathologists might be unaware of the problems physicians have when confronted with a diagnosis that deviates from established nomenclature. Therefore, careful evaluation of the pathology report and thorough multidisciplinary meetings with the pathologists may aid physicians to come to an optimal treatment for patients possibly having LCNEC (**chapter 4**).

2. The clinical presentation of LCNEC and evaluation of optimal treatment

2.1 Occurrence of LCNEC in the Netherlands

The occurrence of LCNEC has increased with over 2-fold in the last two decades in the Netherlands to approximately 150 patients yearly. This accounts for ±0.9% of all lung cancer patients, but remains to be 2-fold different from the incidence reported by institutional surgical cohorts (3%) (**chapter 3**[3,43]). If this 3% is the 'accurate incidence' of LCNEC, on average 200 patients yearly are misdiagnosed plausibly due to the previously reported diagnostic difficulties (**chapters 5, 6** and **8**). Not only in the Netherlands, but

also in the United States of America an increased recognition of LCNEC is reported[4]. Several possibilities may explain the increase in awareness of LCNEC. First, the increased clinical significance to recognize non-squamous NSCLC using additional immunohistochemical stains because of the introduction of platinum-pemetrexed chemotherapy (2008) and targeted therapy with tyrosine kinase inhibitors (2009). Secondly, the usage of larger (core) biopsies in recent years, enabling a more easily recognition of neuroendocrine morphology being essential to diagnose LCNEC; and finally, the incidence of LCNEC has possibly increased because of a change in smoking behavior from non-filter to filter cigarettes. Filter cigarettes are partly causal to the increased incidence of adenocarcinomas, most likely due to the higher amount of carcinogens being deeply inhaled into the (peripheral) alveoli/bronchioles[44]. More than 90% of patients with LCNEC are (former) smokers. Furthermore, the majority of LCNEC tumors are located near the alveoli/bronchioles, suggesting a role for smoking filter cigarettes in the etiology of LCNEC (**chapter 2** and **chapter 11**).

2.2 Clinical characteristics and treatment of LCNEC

To treat a patient with LCNEC in a similar fashion as what is advised for patients with SCLC disease seems a rational approach based on currently available data (**chapter 2**). Treatment of LCNEC as SCLC is also supported by comparing clinical characteristics in **chapter 3**, where we observed that patients with advanced LCNEC disease had a metastatic pattern and prognosis that was quite similar to patients with SCLC disease. Previous studies have indicated that clinical characteristics such as age of onset, history of smoking and prognosis for LCNEC is similar to SCLC (**chapter 2**). In contrast to these findings, treatment of LCNEC patients in the Netherlands is somewhat different from SCLC as fewer patients with LCNEC received adjuvant chemotherapy after surgery (23% *versus* 75%). This lower frequency of adjuvant chemotherapy is also observed in other (European) countries with 14-36%[3,45-49]. Furthermore, LCNEC patients were also treated less frequently with chemotherapy than SCLC patients in stage IV disease, possibly because of expected resistance to chemotherapy in LCNEC or because treatment guidelines were unclear[5]. Nonetheless, the type of chemotherapy treatment shifted from mainly platinum-gemcitabine ('NSCLC type') towards platinum-etoposide ('SCLC type') from 2003 to 2012. SCLC type chemotherapy was also favored amongst physicians (>80%) as was shown by the results from a nationwide survey (2014, **chapter 9**). Exact causes for the observed shift from NSCLC type to SCLC type chemotherapy are unclear; until 2015 LCNEC was not taken considered in treatment guidelines[5]. A small study, but published in a high impact journal (JCO), might have directed treatment of LCNEC more towards SCLC since it reported an improved response rate when SCLC type chemotherapy was given[45].

Contrary to the support for SCLC type chemotherapy treatment in the Netherlands, we observed that NSCLC type chemotherapy might lead to a better outcome in patients with LCNEC, specifically platinum-gemcitabine chemotherapy (chapter 9). Thus far three studies compared NSCLC *versus* SCLC type chemotherapy; the most recent studies supported the use of chemotherapy regimen other than SCLC type[11,50,51]. These recent results contradict the current expert opinion advice stated in the American Society for Clinical Oncology (ASCO) guideline that supports SCLC type treatment[5]. An evaluation of the literature for all published metastatic LCNEC cohorts treated with first-line chemotherapy (n=7) highlights the different results found in LCNEC, likely due to relatively low number of patients analyzed [11,45,50-54]. Based on currently available data, a future randomized trial would ideally compare cisplatinum-gemcitabine (or paclitaxel) *versus* cisplatinum-etoposide chemotherapy. But considering the rarity of LCNEC, together with the difficulties to diagnose LCNEC on biopsy specimen, and the large costs related to clinical trials, a prospective evaluation incorporated into a nationwide registry and by patient centralization may be a more feasible option.

3. Molecular alterations identified in LCNEC and related clinical relevance

3.1 Commonly (in)activated pathways in LCNEC

Studies from the 90's and early 2000 have already emphasized that LCNEC and SCLC have striking molecular similarities. For example, frequent aberrations in the *RB1* pathway, regulating the cell cycle and causing uncontrolled cell proliferation, and the *TP53* pathway, enabling cells to escape from apoptosis, have been reported. However, not all LCNEC and SCLC clustered and subgroups were identified using gene expression analyses, suggesting the existence of genomic different LCNEC/SCLC subtypes[55,56]. More recently, new techniques have enabled high-throughput genomic analysis of LCNEC by whole exome sequencing or (high coverage) exon targeted sequencing[24,36,38,57,58].

Activating mutations or translocations/amplifications in LCNEC are rare and therefore a role for targeted therapy currently remains limited (chapter 11). Nevertheless, recent genomic analyses have discovered LCNEC subtypes such as the bi-allelic *TP53* and *RB1* gene inactivated LCNEC, similar to the hallmarks of SCLC (chapter 11)[24,38,59]. Also, a bi-allelic *STK11* and/or *KEAP1* gene inactivation with *RB1* wild-type LCNEC subtype was identified. Possibly, *CDKN2A* (p16) inactivation is also mutually exclusive to mutations in *RB1* but this requires further investigation[38,60]. The clinical relevance of all these identified subtypes remained unclear in literature; however, we provide support for

application of these LCNEC subtypes in clinical practice (**chapter 10**). Specifically, *RB1* wild-type LCNEC treated with NSCLC type chemotherapy (platinum-gemcitabine or paclitaxel) may improve patient outcome when compared to the SCLC type (platinum-etoposide) chemotherapy. The frequency of *RB1* mutations identified in our study (47%) was slightly higher compared to the previous cohorts reported, but similar to another stage IV LCNEC cohort (44%) possibly indicating that *RB1* is more often affected in metastatic LCNEC (30% *versus* 45%)[61]. Finally, in a cohort of SCLC patients with *RB1* wild-type and/or loss of RB1 IHC, a poor response to SCLC type chemotherapy was recently described[62]. Hence, it is plausible that IHC expression of RB1 may be used in the near future as a marker in high-grade neuroendocrine carcinoma for chemotherapy decision.

3.2 (New) Systematic treatment options for LCNEC

Several targets of treatment could be of interest for LCNEC in the near future as described in **chapter 11**. Evidence for clinical activity was recently provided for a humanized anti-DLL3 monoclonal antibody-drug conjugated to a DNA-damaging pyrrolobenzodiazepine dimer toxin[63,64]. Response was correlated with immunohistochemical expression of DLL3 antibody in SCLC. Currently, the evaluation of DLL3 being expressed in our series of LCNEC is ongoing.
Additionally, immunotherapy (PD-L1 and PD1 inhibitors) has recently been established as second line treatment for NSCLC and is currently being investigated in SCLC[65-68]. A recent study has investigated PD-L1 expression in early stage LCNEC showing expression in up to 15% of cases[69]. The mutational burden, previously correlated to response to immunotherapy[70], is extremely high in LCNEC (±10/mb) and therefore this tumor may show favorable response to immunotherapy[24,38]. Currently we are evaluating all LCNEC for PD-L1 expression (Dako 28-8 antibody) to explore potential benefits of PD-L1 inhibition related to the genomic LCNEC subtypes.

4. Future perspectives

The studies described in this dissertation provide additional insights into the diagnosis and treatment of metastatic LCNEC and combined new genomic insights with clinical relevant treatment. Together with (emerging) therapies, these results may enable an improved patient management in the near future. If considered feasible, future research should include the initiation of a randomized multi-center clinical trial for patient with high-grade disease established by mitosis (>10 / 2mm^2) or Ki-67 ≥20%, with neuroendocrine morphology or undifferentiated morphology with ≥2 neuroendocrine IHC marker staining and with non-small cell cytological features. Eligible patients will be

randomized for cisplatinum-gemcitabine *versus* cisplatinum-etoposide chemotherapy treatment with IHC RB1 as stratification factor (positive (≈35%) versus negative). Next to this, studies are needed that evaluate chemotherapy drug combinations (i.e. different NSCLC type regimens *versus* SCLC type regimens) in LCNEC with functional and inactivated *RB1* gene, preferably in mouse models and/or cell-lines. These analyses should be combined with inhibitors, including but not limited to, $p16^{Ink4A/CDKN2A}$, CDK4 and CDK6. Positive results from any of these two suggested studies would bring personalized treatment for high-grade neuroendocrine carcinomas including LCNEC, a step closer to clinical practice.

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General summary

Pulmonary neuroendocrine tumors, except for the small cell lung cancer (SCLC) subtype, comprehend a rare subcategory of lung cancers not often encountered in clinical practice (±5%)[1,2]. Large cell neuroendocrine carcinoma (LCNEC), the non-small cell subtype of the high-grade neuroendocrine carcinomas, is considered to have an incidence of approximately 3%[3], and presents usually with distant metastasis at diagnosis[4]. To establish a LCNEC diagnosis, both observation of non-small cell cytological features with high-grade disease (mitosis >10 / 10 high power fields and abundant necrosis), and neuroendocrine morphology with neuroendocrine differentiation confirmed by immunohistochemistry (IHC) staining is required[5,6]. The clinical characteristics of LCNEC are further described in **chapter 1 and 2**. In current clinical practice, pathologists and clinicians come across several problems regarding the diagnosis and treatment of LCNEC[7,8]; with the two biggest difficulties being:

I. Pathologist may find it difficult to diagnose LCNEC because of overlapping diagnostic classification criteria with SCLC [9-13] and the lack of diagnostic criteria for LCNEC on a biopsy specimen[14-18].
II. It is difficult for clinicians to choose optimal chemotherapy treatment for advanced disease LCNEC as adequate comparative studies and treatment guidelines are lacking[19].

Therefore, temporal changes in the diagnosis and treatment of LCNEC in the Netherlands were investigated and furthermore, we aimed to optimize diagnosing of LCNEC on a biopsy specimen and select the best treatment for LCNEC when the disease has already metastasized (i.e. chemotherapy treatment). For this final aim, we combined the experience of genomic profiling in LCNEC of others (Fernandez-Cuesta et al.)[20] together with our knowledge on chemotherapy treatment outcome. For these analyses, we used comprehensive databases from the Netherlands Cancer Registry (IKNL) and the nationwide pathology registry (PALGA) which together have data on treatment and tumor diagnosis of (±1000) patients with LCNEC that were diagnosed between 01-01-2003 to 31-12-2012. Finally, precise diagnoses of LCNEC were ensured by reviewing a substantial amount of LCNEC cases by a panel of pathologists.

1. Summary of observations relevant for the establishment of a LCNEC diagnosis

1.1 Incidence and diagnosis of LCNEC

An evaluation of the cancer registry database in **chapter 3** showed that the frequency of patients diagnosed with LCNEC increased with over 2-fold from 2003-2009 *versus* 2010-2012, more frequent in metastatic disease (>55%). In 2012, approximately 150 patients

were diagnosed with LCNEC. Likewise, in **chapter 5** we show that LCNEC was diagnosed on a biopsy specimen 2.5-fold more often comparing 2003-2007 *versus* 2008-2012. In 56% (n=546) of LCNEC diagnoses, the diagnosis was established on a biopsy or cytology specimen regardless of the fact that the World Health Organization classification (WHO, 2004-2015) recommends diagnosing LCNEC on surgical resection specimen only. Comparing ≤2007 *versus* ≥2008, diagnosis of LCNEC on biopsy/cytology specimen increased from 43% (n=120) to 62% (n=326, *P*<0.001) of all LCNEC diagnoses.

1.2 Nomenclature usage and description of WHO criteria in LCNEC

In **chapter 4**, an exemplary patient case is provided emphasizing the clinical relevance of an accurate diagnosis in patients with possible LCNEC. In this puzzling diagnostic situation, vague (non-WHO) nomenclature was often used. Hence, in **chapter 5** we evaluated summaries of pathology reports of all patients diagnosed with a pulmonary neuroendocrine tumor (n=3052) and observed that this non-WHO nomenclature is often (20%) used to describe a diagnosis of neuroendocrine carcinoma not being SCLC (n=1316). This non-WHO nomenclature was established more often on a biopsy specimen (62%) *versus* a resection specimen (26%). Importantly, this nomenclature was confusing for clinicians, which possibly could have led to a different treatment plan. The description of the WHO diagnostic classification criteria for LCNEC was evaluated in **chapter 6**. We screened 882 of full LCNEC pathology reports. In 71% of the cases, the diagnostic WHO criteria for LCNEC were not or vaguely described; neuroendocrine morphology and the mitotic index lacked in 44% and 86% of reports, respectively. Also, the lack of morphology description and mitotic index was more frequent on biopsy specimens (60% and 94%), compared to surgical resections (40% and 80%, both *P*=0.001). Of 210 LCNEC cases, the original histological slides were obtained for panel review by pathologists and a diagnosis different from LCNEC was established in 30%, for example non-small cell lung cancer (NSCLC), SCLC, and carcinoid. Nevertheless, there was no significant difference in the frequency of diagnosis other than LCNEC in reports not describing all required criteria (33%, n=53) compared to reports that completely followed the WHO classification (23%, n=11; *P*=0.14)

1.3 Diagnostic overlap in LCNEC diagnoses on biopsy specimen

Finally, in **chapter 8**, we compared all available pre-operatively taken biopsy specimens from the identical anatomical location were LCNEC was diagnosed on a resection specimen (n=110). LCNEC was diagnosed pre-operatively in 22% compared to 47% by panel review. Other diagnoses included NSCLC (42% & 44%), SCLC (16% & 0%) and carcinoid (6% & 3%). In 50% of these biopsies neuroendocrine morphology was absent

or the biopsy specimen did not allow for an adequate evaluation. In such cases, staining for ≥2 out of three neuroendocrine immunohistochemical markers (CD56, chromogranin-A and/or synaptophysin) was useful to separate LCNEC from NSCLC, thereby improving identification of LCNEC pre-operatively from 47% to 93%. To incorporate staining of neuroendocrine markers in tumors without neuroendocrine morphology (i.e. undifferentiated NSCLC) is subject of debate as shown in chapter 7. Nevertheless, based on the presented data we propose a slight adjustment to the current WHO classification as illustrated in chapter 8.

2. Summary of observations relevant for the treatment of patients with LCNEC

2.1 Treatment in all stages of LCNEC

In chapter 2 & 3 we review and compare clinical characteristics such as age, tumor location and prognosis, of patients with LCNEC (n=952) *versus* those with NSCLC (n=43,886) and SCLC (n=11,844). It was concluded that the presentation of LCNEC is relatively similar to that of NSCLC early (local) disease; however, in metastatic disease this is more comparable to SCLC. If a patient with LCNEC is treated in a similar fashion as a patient with SCLC disease, the overall survival (OS) would be similar to SCLC but worse than NSCLC patients. Notably, patients with early stage LCNEC more often receive surgical treatment for the primary tumor than SCLC patients (87% *versus* 19%, *P*=0.01) but also receive less frequent (adjuvant) chemotherapy treatment (23% *versus* 75%, *P*=0.01).

2.2 Treatment of metastatic LCNEC

In chapter 3 and chapter 9 it is shown that chemotherapy is given less often to patients with metastasized LCNEC (38%) *versus* SCLC (62%, *P*=0.01) patients. Chemotherapy treatment has changed over the past few years for LCNEC. A platinum-etoposide combination (i.e. 'SCLC type') is given more frequently to patients with LCNEC disease increasing from 31% (2003-2009) to 53% (2010-2012, *P*=0.002). Yet, we also found a treatment advantage for 'NSCLC type' chemotherapy, including either platinum-gemcitabine or platinum-taxane with an OS of 8.5 (95% confidence interval (CI), 7.0–9.9) months, *versus* SCLC type chemotherapy with an OS of 6.7 (95% CI, 5.0–8.5) months (hazard ratio (HR) 1.66 (95% CI, 1.08–2.56); *P*=0.020) by retrospective evaluation of all patients with metastatic first-line chemotherapy treated panel-consensus reviewed LCNEC (n=128). Furthermore, patients treated with platinum-pemetrexed chemotherapy

had a worse OS of 5.9 (95% CI, 5.0–6.9) months compared to NSCLC type chemotherapy (HR 2.51 (95% CI, 1.39–4.52); *P*=0.002).

3. Summary of relevant findings from molecular evaluation of LCNEC

In **chapter 11** the available genomic studies on LCNEC were reviewed to provide a comprehensive overview of relevant molecular alterations and future targets of treatment. Additionally, we investigated if the subtypes described in **chapter 11**, have an implication on chemotherapy treatment (**chapter 10**). Treatable driving molecular aberrations such as *EGFR* mutation or *ALK* rearrangement occur in less than 1% of LCNEC, with few case reports showing beneficial effects of targeted treatment. The recently identified LCNEC subtype having bi-allelic inactivation of *TP53* and *RB1* (30-45%) genes may be of interest as well as the subtype recognized by inactivation of *STK11/KEAP1* genes (30-40%) and/or *RB1* wild-type. In **chapter 10**, a total of 79 LCNEC tumors of chemotherapy treated patients were evaluated by next generations sequencing for the relevant genes. *RB1* mutations were detected in 47% and were mutually exclusive with mutations in *STK11*. Patients with LCNEC *RB1* wild-type had superior survival when treated with NSCLC type chemotherapy (platinum-gemcitabine or taxanes) *versus* SCLC type chemotherapy (platinum-etoposide) OS 9.6 [95% CI 7.7-11.6] months *versus* 5.8 [5.5-6.1] months, p=0.026. Furthermore, RB1 protein expression was evaluated in 109 LCNEC; loss of RB1 protein expression was identified in 72% and OS compared for chemotherapy type showed again favorable effects for NSCLC type *versus* SCLC type (p=0.001). Identical results were found for analysis of the progression free survival.

In **chapter 12** the results obtained during our research are discussed and a more global overview is provided for the diagnosis, treatment and recently described molecular background of LCNEC. Collectively, the new insights as written in this thesis can improve the diagnosis and treatment of LCNEC and provides guidance for a randomized clinical trial in the near future.

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Nederlandse samenvatting

Neuro-endocriene tumoren van de long, met uitzondering van het kleincellige longkanker (SCLC) subtype, komen maar zelden voor in Nederland (±5%)[1,2]. Een vorm van deze zeldzame longkanker is het grootcellig neuro-endocriene carcinoom (LCNEC). Dit is een zeer agressieve sneldelende tumor (hooggradig) met een niet-kleincellig morfologie. De gerapporteerde incidentie van LCNEC is ongeveer 3%[3] en de diagnose wordt meestal pas gesteld bij ziekte op afstand wanneer de kanker al uitgezaaid is (metastase)[4].

De klinische kenmerken van LCNEC worden beschreven in hoofdstuk 1 en 2, de diagnose van LCNEC kan echter pas definitief worden vastgesteld wanneer bij pathologisch onderzoek op een chirurgisch verkregen stuk tumorweefsel de volgende tumor-kenmerken worden gevonden: i) cytologische eigenschappen van een niet-kleincellige tumor, ii) een hooggradige tumor op basis van mitose (>10 per 10 gezichtsvelden van 40x, en grote zones van necrose) , iii) een neuro-endocriene morfologie en iv) neuro-endocriene differentiatie bevestigd door immunohistochemische kleuringen (IHC)[5,6]. Dit klinkt erg overzichtelijk maar in de huidige klinische praktijk worden zowel pathologen als (long)oncologen met verschillende problemen met betrekking tot de diagnose en behandeling van LCNEC geconfronteerd[7,8].

De twee voornaamste problemen kunnen als volgt worden samengevat:

I. Door het ontbreken van diagnostische criteria voor LCNEC op een biopsie-preparaat[9-13], en overlappende diagnostische classificatie criteria met SCLC[14-18], kan het lastig zijn de diagnose LCNEC te stellen in de dagelijkse kliniek.
II. Doordat er onvoldoende vergelijkende studies zijn verricht, ontbreekt een duidelijk advies vanuit een consensus richtlijn voor systemische behandeling van patiënten met gemetastaseerde LCNEC[19]. In de huidige praktijk is de behandeling voor deze patiënten onduidelijk en niet gestandaardiseerd.

Aan de hand van deze probleemstellingen zijn in het kader van mijn promotie-traject tijdelijke veranderingen in de diagnose en behandeling van LCNEC in Nederland onderzocht. Daarnaast hebben we bekeken of de diagnose van LCNEC op een biopt kan worden geoptimaliseerd, bijvoorbeeld door het toevoegen van additionele immuno-histochemische kleuringen. Vervolgens is onderzocht welke vorm van chemotherapie behandeling leidt tot een toename van overleving en progressie vrije overleving in de eerstelijnsbehandeling. Deze analyses werden verricht met behulp van beschreven moleculaire profielen van LCNEC (Fernandez-Cuesta et al.) [20] en deze te combineren met data uit een tweetal uitgebreide databanken van het Nederlandse Kankerregister (IKNL) en het Pathologisch Anatomische Landelijk Geautomatiseerd Archief (PALGA).

Gecombineerd gaven deze twee databanken informatie over de behandeling en diagnose van ± 1000 patiënten met LCNEC die tussen 01-01-2003 en 31-12-2012 werden gediagnosticeerd. Tenslotte werd de precisie van de diagnosen geoptimaliseerd door een aanzienlijk aantal tumoren door een panel van pathologen te laten reviseren.

1. Samenvatting van de waarnemingen die relevant zijn voor de diagnose LCNEC

1.1 Incidentie en diagnose van LCNEC

Uit een evaluatie van de databank van het IKNL in **hoofdstuk 3** bleek dat de frequentie van patiënten gediagnosticeerd met LCNEC met meer dan 2 keer toenam van 2003-2009 t.o.v. 2010-2012; nog veel vaker wanneer er sprake was van metastasen (>55%). In 2012 werden ongeveer 150 patiënten gediagnosticeerd met LCNEC in Nederland. In **hoofdstuk 5**, op basis van de PALGA databank, beschrijven we dat LCNEC 2.5 keer tegenwoordig vaker is gediagnosticeerd op een biopsiepreparaat (2003-2007 t.o.v. 2008-2012). In meer dan de helft (56% (n=546)) van de vastgestelde LCNEC-diagnoses werd de diagnose vastgesteld op een biopsie- of cytologisch preparaat, ongeacht het feit dat de classificatie van de Wereldgezondheidsorganisatie (WHO, 2004-2015) stelt dat de diagnose LCNEC uitsluitend op chirurgisch verkregen weefsel kan worden gesteld. Wanneer de tijdsperiode ≤2007 wordt vergeleken met ≥2008, dan steeg de diagnose van LCNEC gesteld op een biopsie / cytologie-preparaat van 43% (n=120) tot 62% (n=326, *P*<0,001).

1.2 Nomenclatuurgebruik en beschrijving van WHO-criteria in LCNEC

In **hoofdstuk 4** wordt een patiënt gepresenteerd waarin de klinische relevantie van een nauwkeurige diagnose wordt onderstreept bij patiënten met mogelijke LCNEC. In deze complexe diagnostische casus werd vaak vage (niet-WHO) nomenclatuur gebruikt. Daarom hebben we in **hoofdstuk 5** samenvattingen van pathologieverslagen van alle patiënten gediagnosticeerd met een neuro-endocriene longtumor (dus niet alleen LCNEC maar alle subtypen) geëvalueerd (n=3052) en werd vastgesteld dat deze niet-WHO-nomenclatuur vaak (20%) wordt gebruikt om een diagnose van neuro-endocrien carcinoom (n=1316) te beschrijven (m.u.v. SCLC). Niet-WHO-nomenclatuur werd vaker op een biopsie preparaat (62%) t.o.v. een chirurgisch verkregen tumor preparaat (26%) toegepast. Belangrijk was dat deze nomenclatuur verwarrend was voor artsen, en dat dit mogelijk tot een niet optimaal behandelingsplan kan leiden doordat de diagnose dan door clinici anders geïnterpreteerd kan worden.

In **hoofdstuk 6** werd de beschrijving van de WHO diagnostische classificatie criteria voor LCNEC geëvalueerd. In totaal werden 882 LCNEC-pathologie rapporten geanalyseerd. In 71% van de gevallen waren de diagnostische WHO-criteria voor LCNEC niet of onduidelijk beschreven; neuro-endocriene morfologie en de mitotische index welke volgens de richtlijn minimaal beschreven moeten worden, ontbraken in respectievelijk 44% en 86% van de rapporten. Gebrekkige beschrijving van neuro-endocriene morfologie en de mitose index kwam vaker voor op biopt preparaten (60% en 94%), in vergelijking met chirurgische verkregen tumor preparaten (40% en 80%, beide *P*=0.001). Van 210 LCNEC diagnoses uit heel Nederland werden de oorspronkelijke histologische coupes verkregen voor revisie door een panel van pathologen. In 30% werd de diagnose 'anders dan LCNEC' gesteld, bijvoorbeeld niet-kleincellige longkanker (NSCLC), SCLC of een carcinoid. Niettemin was er geen significant verschil in de frequentie van de diagnose anders dan LCNEC in rapporten die niet alle vereiste WHO-criteria hadden beschreven (33%, n=53) in vergelijking met rapporten die de WHO-classificatie volledig beschreven (23%, n=11; *P*=0.14)

1.3 Diagnostische overlap in LCNEC diagnoses op biopsie preparaten

Tenslotte werden in **hoofdstuk 8** alle beschikbare preoperatief afgenomen biopsie preparaten en het daarna afgenomen chirurgische resectie preparaat, van een identieke anatomische locatie, met de diagnose LCNEC met elkaar vergeleken (n = 110). LCNEC werd preoperatief gediagnosticeerd in 22% vergeleken met 47% vastgesteld door het revisie panel van pathologen. Andere diagnoses gesteld op het preoperatief biopt preparaat waren NSCLC (42% en 44%), SCLC (16% en 0%) en carcinoid (6% en 3%). In 50% van deze preoperatieve biopten was de neuro-endocriene morfologie, die later wel geobserveerd werd op het resectie preparaat, afwezig. Anderzijds lieten de biopsie preparaten een adequate evaluatie van de (neuro-endocriene) morfologie soms niet toe. In dergelijke gevallen was het toepassen van een cutoff van neuro-endocriene IHC-marker aan kleuring van ≥2 van de drie markers (CD56, chromogranine-A en/ of synaptofysine) nuttig om LCNEC van NSCLC te onderscheiden. Retrospectief toepassen van deze cutoff, zou de identificatie van LCNEC preoperatief van 47% tot 93% hebben verbeterd. Het toepassen van neuro-endocriene markers in tumoren zonder neuro-endocriene morfologie (d.w.z. ongedifferentieerde NSCLC) is volgens de huidige WHO-classificatie discutabel, zoals bediscussieerd in een letter to the editor in **hoofdstuk 7**. Desalniettemin stellen we, op basis van de gepresenteerde gegevens, een lichte aanpassing aan de huidige WHO-classificatie voor, verder geïllustreerd in **hoofdstuk 8**.

2. Samenvatting van de waarnemingen die relevant zijn voor de behandeling van patiënten met LCNEC

2.1 Behandeling in alle stadia van LCNEC

In de **hoofdstukken 2 en 3**, worden de klinische kenmerken zoals leeftijd, tumor locatie en prognose vergeleken van patiënten met LCNEC (n=952) versus die met NSCLC (n=43.886) en SCLC (n=11.844). Op basis van deze gegevens concludeerde wij dat de klinische kenmerken van LCNEC relatief gelijk is aan die van NSCLC bij een vroege (lokale) ziekte. Echter, wanneer de ziekte al is gemetastaseerd, dan zijn deze kenmerken vergelijkbaar met SCLC. Als een patiënt met LCNEC op een vergelijkbare manier zou worden behandeld als een patiënt met SCLC, dan zou de ziekte overleving vergelijkbaar zijn met SCLC, maar slechter t.o.v. patiënten met NSCLC. In Nederland werden patiënten met een vroeg ziektestadium van LCNEC vaker chirurgische behandeld dan patiënten met SCLC (87% versus 19%, *P*=0.01). Echter, patiënten met LCNEC krijgen ook minder frequente (adjuvante) chemotherapiebehandeling (23% versus 75%, *P*=0.01).

2.2 Behandeling van metastatische LCNEC

In **hoofdstuk 3 en hoofdstuk 9** beschrijven we dat chemotherapie minder vaak wordt gegeven aan patiënten met gemetastaseerde LCNEC (38%) dan aan patiënten met SCLC (62%, *P*=0.01). In de afgelopen jaren is de chemotherapie behandeling voor patiënten met LCNEC veranderd. Sinds 2010 wordt de platinum-etoposide chemotherapie combinatie (dat wil zeggen 'SCLC-type') vaker gegeven aan patiënten met LCNEC (31% tussen 2003-2009 t.o.v. 53% tussen 2010-2012, *P*=0.002). Echter, onze gegevens van 128 met eerstelijns chemotherapie behandelde patiënten met gereviseerde LCNEC suggereren een mogelijk behandelvoordeel bij het gebruik van platinum-gemcitabine en platinum-taxaan chemotherapie combinaties (‘NSCLC-type’). De totale overleving voor de NSCLC-type chemotherapie was 8,5 maanden, (95% betrouwbaarheidsinterval (CI), 7.0-9.9) t.o.v. SCLC type chemotherapie met 6,7 maanden (95% CI, 5.0-8.5) wat een Hazard Ratio (HR) geeft van 1.66 (95% CI, 1.08-2.56), *P*=0.020). Bovendien hadden patiënten die werden behandeld met chemotherapie met platinum-pemetrexed, een slechtere overleving van 5.9 (95% CI, 5.0-6.9) maanden t.o.v. de overige NSCLC type chemotherapie (HR 2.51 (95% CI, 1.39-4.52), *P*=0.002). Een prospectieve studie moet uitwijzen of NSCLC (gemcitabine-cisplatinum) chemotherapie inderdaad de overleving van patiënten met LCNEC verbetert in vergelijking met SCLC (etoposide-cisplatinum) type chemotherapie.

3. Samenvatting van relevante bevindingen uit de moleculaire evaluatie van LCNEC

In **hoofdstuk 11** werden de beschikbare moleculaire studies die LCNEC onderzochten gereviewed om een uitgebreid overzicht te geven van relevante moleculaire veranderingen en toekomstige veranderingen voor de behandeling van LCNEC. Daarnaast werd er onderzocht of de moleculaire subtypen van LCNEC beschreven in **hoofdstuk 11** een implicatie hebben voor de eerstelijns chemotherapie behandeling (**hoofdstuk 10**). Behandelbare moleculaire afwijkingen zoals een *EGFR*-mutatie of *ALK* herrangschikking komen voor bij minder dan 1% van alle LCNEC patiënten, met enkele casereports waarin effect van gerichte behandeling is beschreven. De recent geïdentificeerde LCNEC subtype met bi-allelische in activatie van *TP53* en *RB1* (30-45%) genen kan van belang zijn, evenals het subtype dat wordt herkend door in activatie van de *STK11 / KEAP1* genen (30-40%) en / of *RB1* wild-type. In **hoofdstuk 10** werden in totaal 79 LCNEC tumoren van chemotherapie behandelde patiënten geëvalueerd met behulp van next-generation sequencing voor de hierboven beschreven relevante genen. *RB1* mutaties werden gedetecteerd in 47% en kwamen niet voor in combinatie met mutaties in *STK11*. Patiënten met LCNEC *RB1* wild-type hadden een superieure overleving bij behandeling met een NSCLC-type chemotherapie (platinum-gemcitabine of taxanen) t.o.v. SCLC-type chemotherapie (platinum-etoposide) met een overleving van 9.6 [95% CI 7.7-11.6] maanden en 5.8 [5.5-6.1] maanden (*P*=0.026). Bovendien werd *RB1* eiwit expressie geëvalueerd in 109 LCNEC tumoren; verlies van het RB1 eiwit werd geïdentificeerd in 72% van de LCNEC en de overleving vergeleken voor chemotherapie subtypen vertoonde wederom een gunstig overlevingsvoordeel voor NSCLC type t.o.v. het SCLC type behandeling (*P*=0.001). Identieke resultaten werden gevonden voor de analyse van de tumor progressie vrije overleving.
De resultaten beschreven in hoofdstuk 10 suggereren dat moleculaire LCNEC subtypen met een andere vorm van chemotherapie moeten worden behandeld. De onder hoofdstuk 9 gesuggereerde prospectieve studie zou dus idealiter ook stratificeren voor LCNEC subtypen om deze resultaten te kunnen valideren.

In **hoofdstuk 12** worden de resultaten verkregen tijdens dit promotietraject besproken en wordt er een globaal overzicht gegeven voor de diagnose, behandeling en recent beschreven moleculaire achtergrond van LCNEC. Tezamen kunnen de nieuwe inzichten zoals beschreven in dit proefschrift de diagnose en behandeling van LCNEC verbeteren. In de nabije toekomst zou dit kunnen bijdragen aan de initiatie van een grote gerandomiseerde klinische trial waarin chemotherapie behandeling voor LCNEC wordt vergeleken gestratificeerd op basis van de beschreven moleculaire LCNEC subtypen.

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Valorization

Valorization

Lung cancer is a highly aggressive disease causing significant morbidity and mortality worldwide. Lung cancer is yearly diagnosed in approximately 13,000 patients in the Netherlands (2016), and is the fourth most common type of cancer in men and women (IKNL). Pulmonary neuroendocrine tumors other than small cell lung cancer (SCLC), define a rare subgroup of lung cancers including the specific type of large cell neuroendocrine carcinomas (LCNEC) with an incidence of 3%. Patients suffering from LCNEC are known to have one of the worst prognoses in lung cancer. Overall survival for LCNEC is approximately 32 months when the disease has not metastasized and 8 months when it has metastasized at diagnosis.
Despite the first description of LCNEC already in 1991, standardized treatment incorporated into a guideline based on the evaluation of randomized clinical trials and/or meta-analyses for this disease is still lacking. In recent years, several new treatment options have emerged for non-small cell lung cancer (NSCLC) and pulmonary carcinoid, including targeted therapy countering the proliferative effects of driver mutations and/or upregulation of such pathways. Yet, the cornerstone of treatment for metastatic LCNEC remains to be (old) chemotherapy regimens evaluated in small cell lung cancer (SCLC) and/or NSCLC, which are not directly comparable. Amongst other, it is difficult to perform clinical trials regarding treatment in patients with LCNEC because of the depicted difficulty to diagnose this tumor. Concerns are particularly raised on the accuracy and precision of LCNEC diagnosed on a biopsy specimen, while this is of high importance to increase the feasibility of clinical trials evaluating treatment in LCNEC.

Therefore, temporal changes in the diagnosis and treatment of LCNEC in the Netherlands were investigated. In addition, we aimed to optimize the diagnosis of LCNEC on a biopsy specimen with additional markers. Furthermore, treatment of LCNEC when the disease has already metastasized was investigated using the experience of genomic profiling in LCNEC by others and related this experience to our knowledge on chemotherapy treatment outcome. With this approach, we were able to define suggestions regarding improvement on LCNEC diagnosis and treatment, possibly leading to an improved patient outcome in the future.

Relevance for the diagnosis of LCNEC

In chapter 3, it is shown that despite the lack of diagnostic criteria in the World Health Organization classification (2004) for LCNEC on a biopsy specimen, pathologists have been diagnosing LCNEC on biopsy specimen and have been doing this more often over the past years. This is also highlighted by our findings in chapter 4, were we show that

the occurrence of advanced LCNEC disease, usually diagnosed on a biopsy specimen, increased with over 2-fold in the Netherlands comparing 2003-2009 *versus* 2009-2012. In 2012, approximately 160 patients with LCNEC were diagnosed, about 1% of all lung cancer types. In Europe approximately 234,000 patients are yearly diagnosed with lung cancer of which it is estimated that 2340-7020 (1-3%) patients are suffering from LCNEC (white book figure 7d). Therefore, although the findings presented in thesis cannot be translated into a direct societal benefit, the results do emphasize that clinicians (pathologists and oncologists) will encounter LCNEC-patients more frequently. In chapters 4-6 and 8, we evaluated the diagnosis of LCNEC in daily practice. We observed that ambiguous diagnostic nomenclature is often, 20% of all neuroendocrine tumor diagnoses, used to describe diagnoses of neuroendocrine carcinomas not being SCLC on a biopsy specimen. Such nomenclature may be confusing for clinicians and can be interpreted in several ways leading to different treatment options. Also, we identified that although the diagnostic criteria for LCNEC are frequently not described in the original pathology reports (71%), this does not necessarily reflect the quality (accuracy) of the diagnosis. However, in up to 30% of LCNEC diagnoses, a diagnosis other than LCNEC was established by panel revision, including NSCLC, SCLC, and carcinoid diagnoses. Finally, we observed that LCNEC is often not recognized on a biopsy specimen because neuroendocrine morphology is absent or ambiguous to identify in such specimen. Collectively, all observed diagnostic problems regarding LCNEC can lead to suboptimal patient management and increase morbidity and mortality with associated economical costs.

Ways to solve the reported diagnostic problems are proposed in this thesis. 1) we should aim to increase the awareness of the diagnostic problems regarding LCNEC, but not limited to, among pathologists and clinicians. We aimed to do this in the Netherlands by organization of several neuroendocrine tumor workshops during the Pathologen Dagen (2017) and the yearly pulmonary oncology course (Wengen op de Wadden). 2) Implementation of standardized reporting protocols may increase unanimity in diagnostic nomenclature and for this reason the recently introduced national PALGA module for lung biopsies has been evaluated and we have provided feedback for the criteria relevant for LCNEC. 3) in this thesis, an adjustment to the current World Health Organization (WHO) classification is proposed (chapter 8) suggesting further implementation of neuroendocrine markers on biopsy specimen to recognize LCNEC in otherwise undifferentiated NSCLC. And finally, 4) RB1 protein marker staining on a biopsy specimen may be a useful diagnostic tool to separate a subgroup of LCNEC possibly requiring NSCLC type (cisplatinum-paclitaxel/gemcitabine) chemotherapy.

Relevance for the treatment of LCNEC

Treatment of LCNEC is subject of debate, according to expert opinion cisplatinum-etoposide chemotherapy (i.e. 'SCLC type') is the favored treatment for metastatic LCNEC. In chapter 9 we have shown that, based on the thus far worldwide largest cohort of LCNEC patients analyzed, NSCLC type chemotherapy is not inferior to SCLC type chemotherapy and may even have a more favorable outcome. Best treatment outcome was observed for the NSCLC type regimen platinum-gemcitabine and platinum-paclitaxel chemotherapy. To the contrary, platinum-pemetrexed chemotherapy, currently used as first-line treatment for non-squamous NSCLC, showed inferior results in LCNEC. These results are important for patients, clinicians as well as the treatment advice given by (inter) national guidelines, as both platinum-etoposide and platinum-pemetrexed are frequently administered to patients with LCNEC (≥65% in 2012) and this requires further consideration. In chapter 10 and chapter 11 of this thesis, we underline the relevance of the recently identified genomic signatures in LCNEC separating a 'NSCLC type' identified by *RB1* wildtype gene and a 'SCLC type' identified by *RB1* gene mutation. Patient with LCNEC of a NSCLC type (i.e. *RB1* wildtype) showed superior overall survival when treated with a NSCLC type chemotherapy regimen. These results should be considered as hypothesis generating, however, the results are encouraging as they provide a rational for personalized treatment, based on genomic profiles in patients with LCNEC disease additional to well-known and treatable oncogenes (e.g. *EGFR)*.

Summary and future envisioned activities to implement results in daily practice

In summary, this thesis describes several possibilities allowing optimization of the diagnosis of LCNEC on a biopsy specimen and chemotherapy treatment in metastatic disease. Combined these results can be of benefit to patients, clinicians, care financers, and public health services by increasing accuracy of the diagnosis and improving treatment outcomes in patients suffering from LCNEC. These results can also be of interest to pharmaceutical companies investigating (new) drug combinations possibly applicable to LCNEC.

This thesis may provide a reference point. For the results described in this thesis to be of a real benefit to society through application in routine care practice, we should undertake several steps soon:

- A prospective study evaluating the adjusted diagnostic criteria for undifferentiated NSCLC on biopsy specimen should be initiated possibly linking PALGA and IKNL data prospectively.

- A European randomized clinical trial evaluating cisplatinum-gemcitabine (or paclitaxel) *versus* platinum-etoposide chemotherapy should be performed. Included patients could be stratified based on *RB1* status.

- Basic translational research should be encouraged in LCNEC evaluating response of chemotherapy drug combinations in different molecular LCNEC subtypes through establishment of cell lines and mice models.

Dankwoord

Dankwoord

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List of publications

Published original research articles and reviews

Mellema, W. W., Dingemans, A. M., Thunnissen, E., Snijders, P. J., **Derks, J.**, Heideman, D. A., Van Suylen, R., and Smit, E. F. KRAS mutations in advanced nonsquamous non-small-cell lung cancer patients treated with first-line platinum-based chemotherapy have no predictive value, *Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer 2013; 8*, 1190-1195

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pulmonary large cell neuroendocrine carcinoma predict chemotherapy outcome. *Authors contributed equally. (accepted Clinical Cancer Research)

Submitted manuscripts

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Curriculum vitae

Curriculum vitae

Jules Derks was born on the 5th of May 1988 in Nijmegen, the Netherlands. In 2000 he graduated from the Montessori College (Nijmegen, the Netherlands). Following the graduation he started his medical studies at the Faculty of Health, Medicine and Life sciences (FHML) at the University of Maastricht. During his studies Jules performed research on pulmonary oncology under supervision of prof. dr. Dingemans at the departments of Respiratory Diseases and Pathology of the Maastricht University Medical Centre (MUMC+).

This work was continued in 2013, after Jules finished his medical studies and started a PhD project under the supervision of prof. dr. Dingemans and prof. dr. Speel focusing on pulmonary large cell neuroendocrine carcinoma. During this PhD project Jules has extensively analyzed the Netherlands cancer and pathology registries and collected a large cohort of LCNEC tumors for biomarker analysis for which additional funding was attained by a grant from the Dutch Cancer Society (2014). He was honored with a short term research fellowship from the European Respiratory Society (ERS) / European Molecular Biology Organization (EMBO) to visit the International Agency for Research on Cancer (IARC) to perform genomic analyses of LCNEC under supervision of dr. Fernandez-Cuesta. The research performed during his PhD was honored with the first prize for best poster at the European Neuroendocrine Tumor Society (ENETS) conference (2015), first prize during the science day of the MUMC+ and the Lung Cancer award (Stichting Nascholing en Cursorisch Onderwijs). Currently, Jules is working at the department of internal medicine at the Zuyderland Medical Centre (Sittard-Geleen, the Netherlands) under supervision of dr. Buijs. In 2019 he will continue his training in pulmonology at the MUMC+ under supervision of prof. dr. Wouters and dr. Hochstenbag.